Microbial Community Analysis of Restaurant Cutting Boards in Seri Kembangan, Malaysia and the Identification of Factors Associated with Foodborne Bacteria Growth

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LIST OF ABBREVIATIONS

AHL	Acylated homoserine lactones
AI-2	autoinducer-2
AIDS	Acquired immune deficiency syndrome
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BLIS	Bacteriocin-like inhibitory substances
CDT	Cytholethal distending toxin
CFU	Colony forming unit
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
EAggEC	Enteroaggregative *E. coli*
EHEC	Enterohemorrhagic *E. coli*
EIEC	Enteroinvasive *E. coli*
ELISA	Enzyme-linked immunosorbent assay
EPEC	Enteropathogenic *E. coli*
ETEC	Enterotoxigenic *E. coli*
FBB	Foodborne bacteria
FQs	Fluoroquinolones
HA	Haemagglutination
HACCP	Hazard Analysis and Critical Control Point
HAV	Hepatitis A virus
HIV	Human immunodeficiency virus
LAB	Lactic acid bacteria

LC-MS	liquid chromatography-tandem mass spectrometry
MLST	Multilocus sequence typing
MOH	Ministry of Health
MRSA	Methicillin-resistant *Staphylococcus aureus*
OD	Optical density
OTUs	Operational taxonomy units
PCoA	Principal coordinate analysis
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
PPi	Inorganic pyrophosphate
QIIME	Quantitative Insights into Microbial Ecology
qPCR	Quantitative polymerase chain reaction
QS	Quorum sensing
RDP	Ribosomal Database Project
RNA	Ribonucleic acid
rRNA	Ribosomal Ribonucleic acid
RTE	Ready-to-eat
SNP	Single nucleotide polymorphisms
TGGE	Temperature gradient gel electrophoresis
UV	Ultra violet
WHO	World Health Organization

PUBLICATIONS AND CONFERENCES ATTENDED

1. Noor-Azira Abdul-Mutalib, Syafinaz Amin Nordin, Kenji Sakai and Yoshihito Shirai, 2014. An overview of foodborne illness and food safety in Malaysia. *International Food Research Journal 22* (3): 896-901 (IF: 0.12).

2. Noor-Azira Abdul-Mutalib, Syafinaz Amin Nordin, Malina Osman, Natsumi Ishida, Kosuke Tashiro, Kenji Sakai, Yukihiro Tashiro, Toshinari Maeda, Yoshihito Shirai. 2015. Pyrosequencing analysis of microbial community and foodborne bacteria on restaurant cutting boards collected in Seri Kembangan, Malaysia and their correlation with grades of food premises. *International Journal of Food Microbiology* 200, 57-65 (IF: 3.155).

3. Noor-Azira Abdul-Mutalib, Syafinaz Amin Nordin, Malina Osman, Ahmad Muhaimin Roslan, Natsumi Ishida, Kosuke Tashiro, Kenji Sakai, Yukihiro Tashiro, Toshinari Maeda, Yoshihito Shirai. 2015. Microbial community analysis of cutting board (CB) samples collected in Seri Kembangan, Malaysia and the identification of factors associated with foodborne bacteria (FBB) growth (Submitted to *AIMS Microbiology*).

4. [Poster] Noor-Azira Abdul-Mutalib, Syafinaz Amin Nordin, Malina Osman, Natsumi Ishida, Kosuke Tashiro, Kenji Sakai, Yukihiro Tashiro, Toshinari Maeda, Yoshihito Shirai. 2015. Pyrosequencing analysis of microbial community on restaurant cutting boards collected around Seri Kembangan, Malaysia and the identification of foodborne bacteria. The International Conference On Biomedical & Health Sciences Research 2015 (26-27th January 2015). PUTRAJAYA, MALAYSIA.

5. [Oral presenter] Noor-Azira Abdul-Mutalib, Syafinaz Amin Nordin, Malina Osman, Natsumi Ishida, Kosuke Tashiro, Kenji Sakai, Yukihiro Tashiro, Toshinari Maeda, Yoshihito Shirai. 2015. Microbial community analysis of cutting board samples collected in Seri Kembangan, Malaysia and the identification of factors associate with the growth of foodborne bacteria. Symposium on Applied Engineering and Sciences 2015 (23rd-24th November 2015). UPM,SELANGOR, MALAYSIA.

ABSTRACT

Foodborne diseases have been associated with microorganisms like bacteria, fungi, viruses and parasites. Most commonly, the outbreaks take place due to the ingestion of pathogenic bacteria like *Salmonella typhi, Escherichia coli, Staphylococcus aureus, Vibrio cholerae, Campylobacter jejuni,* and *Listeria monocytogens*. The disease usually happens as a result of toxin secretion of the microorganisms in the intestinal tract of the infected person. Usually, the level of hygiene in the food premises reflect the quality of the food item, hence restaurant or stall with poor sanitary condition is said to be the contributor to food poisoning outbreak. In Malaysia, food poisoning cases are not rare because the hot and humid climate of this country is very suitable for the growth of the foodborne bacteria, thus the government implements strict rules to ensure workers and owners of food premises prioritize the cleanliness of their working area. Studies of food safety are important, and the results can give information about the types of microorganisms, and factors that affect their growth. The result is crucial to determine how the spread of foodborne bacteria can be controlled safely and the outbreak can be reduced.

This study adopts the pyrosequencing technique to identify bacteria present on 26 kitchen cutting boards collected from different grades of food premises around Seri Kembangan, Malaysia. The analysis generated 452,401 of total reads of OTUs with an average of 1.4×10^7 bacterial cells/cm^2. *Proteobacteria, Firmicutes* and *Bacteroidetes* were identified as the most abundant phyla in the samples. Taxonomic richness was generally high with >1000 operational taxonomic units (OTUs) observed across all samples. The highest appearance frequencies (100%) were OTUs closely related to *Enterobacter* sp., *E. aerogenes*, *Pseudomonas* sp. and *P. putida*. Several OTUs were identified most closely related to known foodborne pathogens including *Bacillus cereus, Cronobacter sakazaki*, *C. turisensis, Escherichia coli, E. coli* O157: H7, *Salmonella bongori, S. enterica, S. paratyphi, S. typhi, S. typhimurium* and *Yersinia enterocolitica* ranging from 0.005-0.68% relative abundance. The condition and grade of the food premises on a three-point cleanliness scale did not correlate with the bacterial abundance and type. To add, the community composition of FBB based on premise grades also showed no similarity. Regardless of the status and grades, all food premises have the same likelihood to introduce foodborne bacteria from cutting boards to their foods and must always prioritize the correct food handling procedure, in order to avoid an unwanted outbreak of foodborne illnesses.

This study further analyzes the effect that might be created by other microorganisms in order to support or suppress the number of pathogenic bacteria. The analysis found that high bacteria group contains a low percentage of FBB compared to low bacteria group. The results also showed that although premise grade C contains significantly higher numbers of overall bacteria, it did not influence the number of FBB. The combinations of *Clostridiales, Flavobacteriales,*

and *Lactobacillales* in the high bacteria group were found to have significant negative associations with FBB. Despite the finding that *Citrobacter, Enterobacter, Erwinia*, *Klebsiella* and *Pantoea* had significant positive correlation with FBB, the population of these bacteria was small. This study suggested that high background biota and a combination of different bacteria have an advantage in preventing FBB from reaching the infectious dose level thus, considered harmless.

CHAPTER 1

INTRODUCTION

1.1 Preface

Foodborne outbreaks caused by foodborne pathogenic bacteria (subsequently written as foodborne bacteria) have been reported with significant morbidity worldwide and pose risk towards the human population. Concurrently, in the less developed country, diarrhoeal diseases are the primary reason for mortality (Schlundt et al., 2004). According to World Health Organization (WHO) (2007), 1.8 million deaths occurred due to the foodborne outbreak in 2005 alone, but the numbers might be underestimated because most of the cases go unreported. In industrialized countries, foodborne diseases are not rare because 30% of the global population experienced foodborne diseases each year. The United States of America reports that around 76 million foodborne diseases occur annually with 325 000 people hospitalized with 5200 cases of mortality (Buzby and Roberts, 2009).

Foodborne diseases occur due to the ingestion of bacteria, viruses or parasites which multiply in the intestine and cause illness or consumption of non-infectious agents like toxin and chemicals (Linscott, 2011). Common symptoms of foodborne illness are diarrhea which sometimes accompanied with nausea and vomiting. Several factors contribute to foodborne illnesses like lack of self hygiene of food handlers, no clean water supply and unclean environment (Meftahuddin, 2002). Food handlers are the most common source of contamination because they can spread harmful organisms by means of faecal-oral route or skin lesions, as well as unclean kitchen utensils or kitchen counters (Linscott, 2011).

In general, the frequent sources of foodborne outbreaks are meat, dairy products, eggs and vegetables whereas the common agents of foodborne outbreaks are *Salmonella* spp, *Staphylococcus aureus, Escherichia coli and Clostridium perfringens* (Pires et al., 2012). The correct food handling practices in every step of food preparation is essential to avoid contamination from the environments, from food handlers themselves and from rare to cooked or ready-to-eat (RTE) food items.

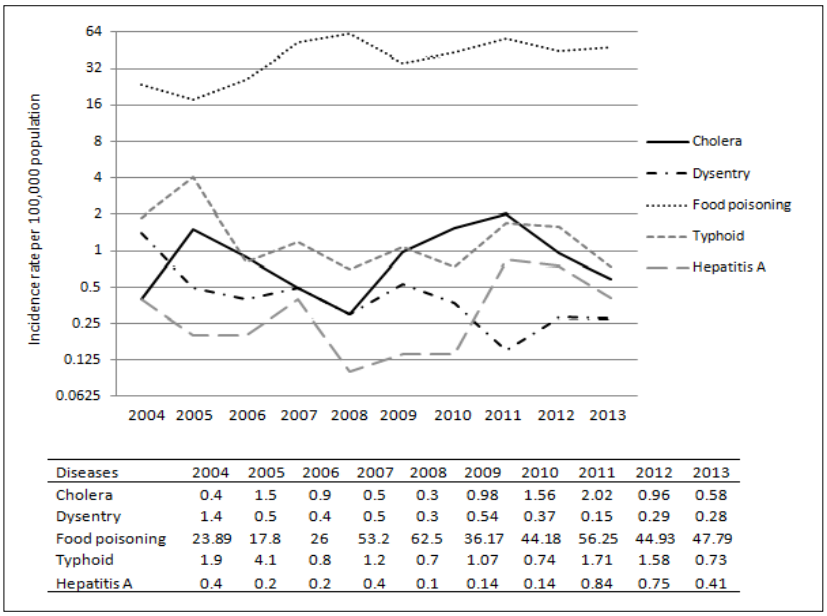
Food handlers are the main food contamination vehicles (Campos et al., 2009), and their level of practice in the kitchen plays an important aspect on the distribution of harmful microorganism from the environment to the food items. Sometimes consumers give prioritization on the price of the food that they purchased instead of the hygiene level of the food premises. Hence, inexpensive and more affordable food; yet pose high risk of contamination become a popular choice. This contaminated food can cause illness to the consumers and might lead to a more serious case like death if appropriate treatment is not given.

1.2 Cases in Malaysia

Malaysia is one of the countries that have high cases of foodborne diseases due to the suitable temperature and condition for the growth of most bacteria. During invasion of pathogens, skin, mucus and intestinal microflora become the first barrier to avoid illness. The last defence mechanism would be the immune system that protect human when the first barrier is affected (Bezirtzoglou and Stavropoulou, 2011). Nevertheless, the immune systems and gut microbial communities depend on human diet which is indirectly influenced by socioeconomic status, culture, population growth and agriculture (Kau *et al*., 2012). This explains why people from different backgrounds and countries have less tolerance towards poor hygiene food prepared locally. It proves that cleanliness is important during food preparation to avoid contamination of food items and illness among local and international consumers. This is because the occurrence of foodborne disease will affect economic growth of the seller as well as the country due to changes in consumers buying patterns (Palma *et al*., 2010).

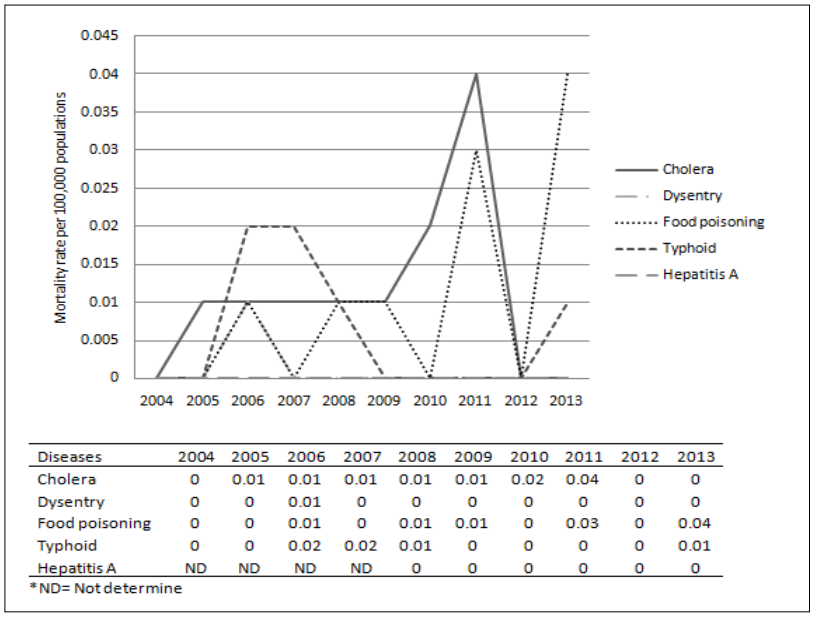
In Malaysia, foodborne diseases are not rare at all. A fact remains that not all cases of food poisoning are reported because most of the affected persons do not seek treatment at the hospital, especially if the cases are not serious. To top it all, before a case can be reported to the authority, a complex of chain called the population exposure must occur first. As an example, a hundred persons eat the same food, then 40 persons become sick and 10 persons went for treatment. After that, the doctor will request for a specimen from some of these patients, and then send them for the analysis in the laboratory. Finally the culture-confirmed case will be reported to the Ministry of Health (Soon *et al*., 2011).

The trends of food and water borne diseases in Malaysia vary over the past few years. There was an increase of cholera, food poisoning and hepatitis A from 2009 to 2011, but a decrease of dysentery. From 2011 to 2013, cases of cholera, typhoid and hepatitis A decrease but dysentery showed an increment. Furtermore, food poisoning cases decrease in 2012 but slightly increase in 2013 (Figure 1.1). The increase of food poisoning cases might indicate that the food handlers have been neglecting the importance of safe food handling in the kitchen. Mortality has been shown to be associated with cholera from 2005 to 2011, but none was reported for the next two years. In 2012 none of the diseases cause mortality, however, in 2013 food poisoning and typhoid was shown to cause death of Malaysian citizen (Figure 1.2).



Diseases	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013
Cholera	0.4	1.5	0.9	0.5	0.3	0.98	1.56	2.02	0.96	0.58
Dysentry	1.4	0.5	0.4	0.5	0.3	0.54	0.37	0.15	0.29	0.28
Food poisoning	23.89	17.8	26	53.2	62.5	36.17	44.18	56.25	44.93	47.79
Typhoid	1.9	4.1	0.8	1.2	0.7	1.07	0.74	1.71	1.58	0.73
Hepatitis A	0.4	0.2	0.2	0.4	0.1	0.14	0.14	0.84	0.75	0.41

Figure 1.1 Incidence rates of food and waterborne diseases (MOH, 2014)



Diseases	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013
Cholera	0	0.01	0.01	0.01	0.01	0.01	0.02	0.04	0	0
Dysentry	0	0	0.01	0	0	0	0	0	0	0
Food poisoning	0	0	0.01	0	0.01	0.01	0	0.03	0	0.04
Typhoid	0	0	0.02	0.02	0.01	0	0	0	0	0.01
Hepatitis A	ND	ND	ND	ND	0	0	0	0	0	0

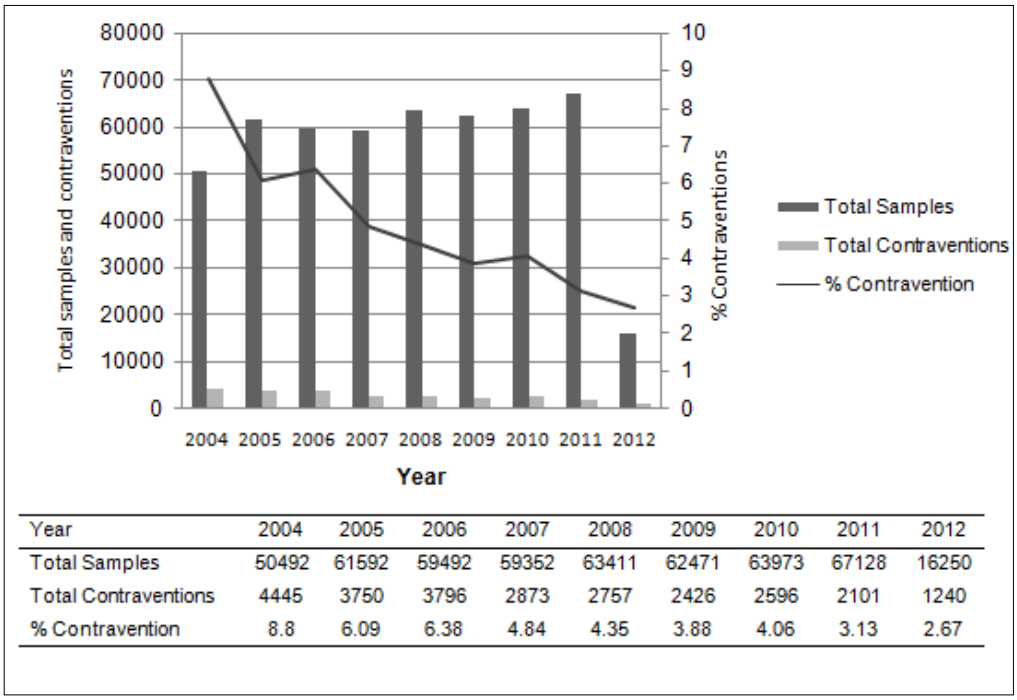
*ND= Not determine

Figure 1.2 Mortality rates of food and waterborne diseases (MOH, 2014)

The Malaysian government has implemented few rules to ensure owners of food premises abide by the rules to avoid illness and outbreak. The hygiene level of the kitchen is the most important aspect that should be given attention because it will reflect the safety of the food to be consumed. In addition, the temperature in the kitchen is usually higher than the dining area which makes it a perfect condition to promote bacterial growth. It has also been proven that foodborne pathogenic bacteria (FBB) can grow on most of the surfaces in the kitchen like cutting board, cloth, sink, cleaning sponge and knives (Kusumaningrum *et al*., 2003; Mattick *et al*., 2003). Cross contamination of food could occur if those items are not properly clean and food handlers neglect the correct way of food preparation.

The main reason for foodborne illness in Malaysia is insanitary food handling procedures which contribute to 50% of the cases (MOH, 2007). It occurs due to preparation of food in advance, inappropriate ways of cooling and insufficient temperature during reheating of food (Beumer and Kusumaningrum, 2003). All of these mishandlings permit the growth of microbial pathogens because they fail to kill the bacteria or they help to keep the bacteria dormant before they reach sufficient temperature to multiply. It is known that temperature play an important part to determine the microbial activity and shelf life of food product (Aung and Chang, 2014) hence, temperature manipulation, as well as hygiene and sanitation of kitchen environment should be the highest priority to ensure safe foods with low risk of contamination are served to consumers.

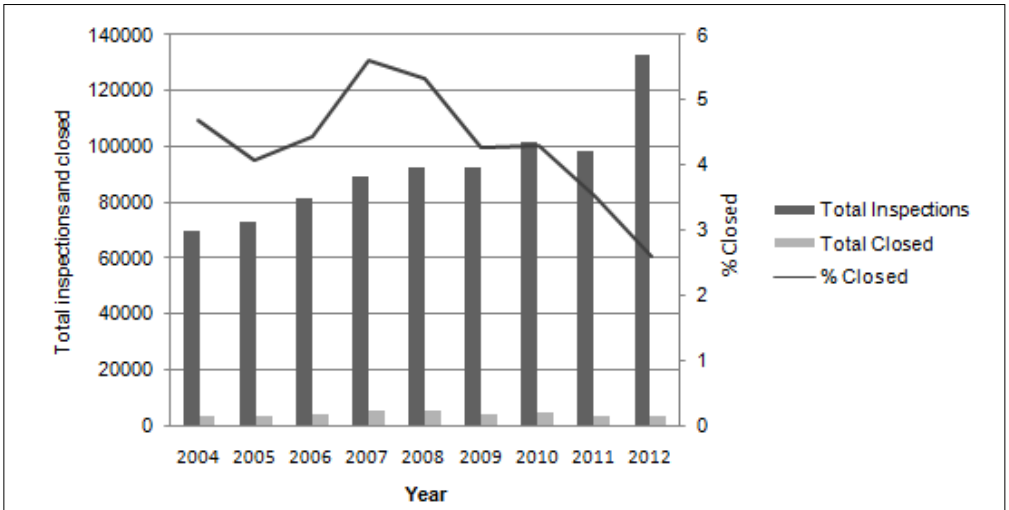
Ministry of Health Malaysia has been collecting food samples from various food premises to determine whether food service establishment prepare food according to the accepted requirement. Each year, more than 10,000 food items were sampled for analysis to determine whether food items are safe for consumption. From 2004 to 2012 the percentage of contraventions was decreasing (Figure 1.3). This shows that as the government enforce the law, food handlers make sure that they always incorporate high level of cleanliness during food preparation, thus reduce the numbers of contraventions.



Year	2004	2005	2006	2007	2008	2009	2010	2011	2012
Total Samples	50492	61592	59492	59352	63411	62471	63973	67128	16250
Total Contraventions	4445	3750	3796	2873	2757	2426	2596	2101	1240
% Contravention	8.8	6.09	6.38	4.84	4.35	3.88	4.06	3.13	2.67

Figure 1.3 Contravention during food sampling between 2004-2012 (Food Safety and Quality Programme, MOH, 2014)

In addition, food premise inspection is also crucial to ensure the level of hygiene in food premises. Around 70000 to 100000 of premises were inspected from 2004-2012. There was an increase in food premises closures from 2005 to 2007 but further decrease until 2012 (Figure 1.4). The increase of food premise closures and law enforcement might motivate owners to boost their premises hygiene level.



Year	2004	2005	2006	2007	2008	2009	2010	2011	2012
Total Inspections	69813	73037	81686	88968	92597	92776	101799	98309	132526
Total Closed	3259	2973	3624	4984	4919	3962	4370	3458	3447
% Closed	4.67	4.07	4.44	5.6	5.31	4.27	4.29	3.52	2.6

Figure 1.4 Food premises inspection and closures 2004-2012 (Food Safety and Quality Programme, MOH, 2014)

The government has implemented laws regarding the hygiene level in the food premises. Under the law, any premise that is categorized as dirty will be sealed and ordered to close down its operations. This is to avoid foodborne outbreak to occur because, apart from getting sick and experience diarrhoea, foodborne diseases can cause mortality and it can easily affect the young, old and immunocompromised person compared to other healthy individuals (Gorman *et al*., 2002). In Malaysia however, most of foodborne diseases are associated with food handlers' insanitary procedure during food manipulation (Soon *et al*., 2011) whereby improper food handling might transfer pathogenic bacteria to food item and cause illness.

The contamination of bacteria to food items is also influenced by several factors like temperature during storage, composition of gases, humidity, interaction between microorganisms and the food, as well as between microorganisms contaminating the food (Hamad, 2012). As consumers, we can always avoid getting foodborne illness by choosing the right food and the right place to dine. In Malaysia, the authority classifies food premises according to the level of cleanliness. Grades like A, B, C and D or "no grade" has been applied to categorize food premise based on their level of sanitation. Several aspects that determine the grades including location, water supply, pest control, ventilation, food storage, toilet room, food handlers' clothing and health condition, food preparation, temperature, and so forth (Food Safety and Quality Programme, MOH, 2013).

In addition to this, the rate of foodborne illness based on premise grades could not be determined. This is because some of the data is treated confidential, therefore, any information regarding the premises that cause the outbreak will

not be disclosed to the public. The information presented is basically general and represent the country as a whole.

1.3 Food premises grading systems

Food premise grades are given by local authorities based on scoring system which reflected to the physical cleanliness of a premise; and the marking system is different from one district to another. Grading systems are based on physical observation of the premises like the location of the garbage bin, water supply, condition of the refrigerator, stock of food etc. Initially, the premises were given 100% mark, and during the inspection, the mark will be deducted based on the contravention or faulty in the kitchen. In general, grade A is given to a clean premise that obtains 76 to 100% marks. The following inspection will be done after a year and if the grade decline, the next inspection time will be done earlier. Grade B is issued to moderately clean food premise that obtains 50 to 75% marks. The subsequent inspection will be carried out in six months. Finally, grade C is given to unclean food premise. This premise will have to close down its operation for two weeks to perform cleaning measures appropriately. After that, another premise inspection will be done to determine whether this premise is competent to run its business again (Ali and Abdullah, 2012).

Grades of food premises will help people by giving an overview of the level of hygiene and sanitation of the food premise. Unacceptable food hygiene level is an indication that eating at the premises will increase the risk of foodborne diseases (Lee *et al*., 2012; Djekic *et al*., 2014). However, studies have proven that incidence of foodborne illness still occurs in a clean and well-known restaurant (Simonne *et al*., 2004; Walczak and Reuter, 2004; Alsop, 2013). Therefore, food handlers are the most important individuals in ensuring that food is always safe for consumption. In addition, it is compulsory for every food handler to attend food handlers training programme held by Food Safety and Quality Division, Ministry of Health, Malaysia. This programme is important to create awareness among food handlers about the significance of personal hygiene and sanitation in the premises (Food Safety and Quality, MOH, 2012).

1.4 Problem statement and significant of study

Ready to eat or prepared food has become a necessity due to the fast pace of living nowadays. The limitation of time for food preparation and rapid urbanization has increase the demand for street foods (Haryani et al., 2007). However, some food premises neglect the importance of hygiene and sanitation and thus, increase the risk of foodborne illness among the consumers. Food handlers play an important role to ensure the safety of food because they are the most common source of contamination. They can spread harmful organisms by means of fecal-oral route or skin lesions, as well as unclean kitchen utensils or kitchen counters (Linscott, 2011). The high microbial load has also been detected on cutting board, counter top, refrigerator and blenders

in school meal services (de Oliveira et al., 2014), which shows an alarming situation involving young generations.

Food contamination is influenced by a lot of factors especially temperature (El-Fadel et al., 2012), and initial microbial load (Jongenburger et al., 2012). Some premises that prepare food in advance do not use a water bath to keep the food hot. They just leave the food on the counter at room temperature, which eventually will favour bacterial multiplication that might harm consumers. Despite the increasing concern of consumers towards restaurant hygiene as a factor where to eat (Ungku Fatimah et al., 2011), restaurant with low hygiene level continue to operate, showing that they still have customers walk into their premises. To top it off, some of these restaurants sell cheaper food and consumers do not experience illness causing them to repeat visit. In addition, there is still a possibility for foodborne illness to occur after dining in clean premises (Simonne et al., 2004, Alsop, 2013) showing that clean environment still allows for contamination to take place.

The population of FBB might be influenced by the microbial diversity of the food or the environment itself. Some bacteria have the ability to support or suppress the growth of FBB. The co-existence of pathogenic bacteria in dairy, meat and seafood industries is of concern for food safety (Gutiérrez et al., 2012). However, the interaction between FBB and good bacteria sometimes result in the reduction of the disease risk due to the production of metabolite that suppresses the growth of FBB. For instance, lactic acid bacteria (LAB) produce bacteriocin-like inhibitory substances (BLIS) as natural antimicrobial agents which increase the safety of the food itself (Cizeikiene et al., 2013). Interaction between LAB and FBB can also reduce toxic material like amine, produced by FBB (Kuley et al., 2013).

The information about the interaction between microorganisms like how they help to reduce pathogenic bacteria or what type of bacteria gives synergistic effect to FBB is important. In addition, the eradication of FBB using normal flora or their metabolites will minimise the usage of chemical or substance that might cause allergies and much safer. The application can be done at any stage of food production and further understanding of their relationship is crucial before it can be executed.

1.5 Research objectives

The objectives of this study were:

1. To determine and compare bacterial community composition in CB samples.
2. To identify and quantify FBB and their association with premise grades.
3. To determine the prevalence of FBB in high and low background biota.
4. To evaluate the ecological correlation of FBB and different phyla, classes, orders and families of other bacteria.
5. To propose precautionary measures based on the findings from this study.

1.6 Hypotheses

The null hypotheses of this study were:

1. There is no association between premise grades and species richness as well as bacterial community composition.
2. There is no association between FBB and the other bacterial phyla, classes, orders and families.
3. There is no association between the FBB and grades of food premises, types, usages and conditions of cutting board.

CHAPTER 2

LITERATURE REVIEW

2.1 Foodborne diseases

The outbreak of foodborne disease is defined as the occurrence of two or more cases of similar illness due to the consumption of food (Soon *et al*., 2011). Foodborne illness can be caused by bacteria, virus and parasite. Most foodborne illnesses like cholera, typhoid fever, hepatitis A, dysentery and food poisoning are associated with acute gastrointestinal symptoms like diarrhea and vomiting (Blackburn and McClure, 2002).

Generally, cholera is a foodborne disease due to the infection of *Vibrio cholera* in the intestinal tract. *V. cholera* can be easily isolated from seafood such as cockles and oysters (Tobin-D'Angelo, *et al*., 2008). Typhoid fever is a disease caused by bacteria called *Salmonella typhi* (Yanagi *et al*., 2009) and the symptoms of infection are fever with body temperature increasing to 39° to 40°C, headache, loss of appetite, and stomach ache. Typhoid fever can easily spread from contaminated food and water or from infected human or carrier (Parry and Beeching, 2009).

Hepatitis is a disease that affects the liver. It is caused by a virus called hepatitis A virus (HAV) that can be transmitted via contaminated food and drink. Dysentery is an infection that leads to severe diarrhea that contains mucus and/or blood in the faeces, vomiting blood or in some serious cases results in death if no treatment is given. An example of organisms that causes dysentery is *Entamoeba histolytica*, a parasite that causes amoebic dysentery (Xun *et al.,* 2009) and *Shigella dysenteriae* that causes bacillary dysentery (Sharma *et al.,* 2010).

2.2 Foodborne pathogens

2.2.1 *Salmonella* spp

Salmonella is a genus of rod-shaped Gram-negative enterobacteria that causes salmonellosis, typhoid fever and paratyphoid fever (Ryan and Ray, 2004). Most of the salmonellosis cases are caused by *Salmonella enteriditis* and *S. typimurium* (Dunkley *et al.,* 2009). *Salmonella* infection is usually caused by ingesting large amounts of the bacteria in contaminated food or water. Generally, salmonellosis occurs when *Salmonella* reaches 10^4 cfu/g. However, cases have been reported with an infective dose of 10-100 cfu/g (Giaccone *et al*., 2012). The main source of *Salmonella* is poultry meat (Carrasco *et al*., 2012) therefore mishandling this type of food such as keep it at room temperature for too long and insufficient cooking temperature can easily cause this bacterium to proliferate and increase the risk of getting sick. In household

environment about 40-60% of salmonellosis occurs due to inadequate handling practices (de Jong *et al*., 2008).

Salmonellosis causes diarrhoea, fever, vomiting, and abdominal cramps 12 to 72 hours after infection. In most cases, the illness lasts 3 to 7 days but most affected persons would recover without treatment (Dworkin *et al.,* 2001). Typhoid fever is characterized by a sustained fever as high as 40°C, profuse sweating, gastroenteritis, and non-bloody diarrhoea. A rash may also appear. Paratyphoid fever resembles typhoid fever but presents a more abrupt onset, milder symptoms and a shorter course. Some very rare symptoms are psychosis (mental disorder), confusion and seizures (Harman and Mason, 2002).

Research has been shown that bacteria possess the ability to mutate and generate different strains with different types of growth condition. One example is *Salmonella spp.,* which is usually linked to the contamination of poultry. One of the strains in this genus is *Salmonella enteritidis* phage Type (PT) 4 which usually relate to the contamination of shell egg and poultry. They can reproduce in the intestinal tract of hens, contaminate the egg before its hardened and survive in the hens' egg (Gantois *et al*., 2008). Thus this can cause illness for consumers if the egg is not properly cooked.

2.2.2 *Escherichia coli*

Escherichia coli are Gram-negative bacteria that usually reside in the lower intestine of warm-blooded animals. Most *E. coli* strains are harmless, but some serotypes like O157: H7 can cause serious food poisoning in humans (Vogt and *Dippold*, 2005). A very low number of *E. coli* O157: H7 has been reported to cause illness. Infective dose of 0.04 cfu/g is set to be the baseline for food safety (FSCJ, 2011) and more than 10^2 cfu/g of this bacterium is considered dangerous to be consumed (Little *et al*., 2001). Enteric *E. coli* are classified on the basis of serological characteristics and virulence properties. Virotypes include: Enterotoxigenic *E. coli* (ETEC), Enteropathogenic *E. coli* (EPEC), Enteroinvasive *E. coli* (EIEC), Enterohemorrhagic *E. coli* (EHEC) and Enteroaggregative *E. coli* (EAggEC) (Todar, 2012). All of them are causative agents of diarrhea; only differing in their way of invasion.

Escherichia coli O157: H7 is an enterohemorrhagic strain of the bacterium. *E. coli* O157 can survive for a longer period on hands, cloths or utensils, which can lead to cross-contamination if bad hygiene practices are followed (Kusumaningrum *et al*., 2003). Hence, the most importance approach to avoid foodborne illness is to ensure hygiene and sanitation are applied in the kitchen area.

Escherichia coli especially strains O157: H7 is the most significant cause of foodborne illness. Previously only one strain is known to cause foodborne disease. However, groups O26, O103, O111 and O145 have been found to be responsible for infection as well (Newell *et al*., 2010; Wick *et al*., 2005; Wu *et al*., 2008). Cattle and ruminants are primary reservoirs for these bacteria, and

improperly cooked meat or contamination through their faeces can cause illness (Fratamico *et al*., 2011). This might happen during slaughtering, packaging, preparation and serving.

2.2.3 *Staphylococcus aureus*

S. aureus is a leading cause of gastroenteritis (Bhatia and Zahoor, 2007). They are Gram-positive, cocci, and formed in grape-like clusters of bacteria (Ryan and Ray, 2004). *Staphylococcus* spp. are facultative anaerobes which are capable of growing in both aerobic and anaerobic conditions. *S. aureus* has no specific nutritional and environmental requirements. However, it has the ability to grow at a_w of 0.86, with pH above 4.8 and temperature as low as 8 or 9°C (Normanno *et al.,* 2005).

Some strains of *S. aureus* also produce an enterotoxin that causes gastroenteritis. Affected person usually recover in 8 to 24 hours and the symptoms include nausea, vomiting, diarrhea, and abdominal pain. Food poisoning usually occurs when the food or the ingredient in the food is contaminated with *S. aureus* and then, is exposed to temperatures that allow for their multiplication.

Foods that are usually contaminated with *S. aureus* are milk, cream, cream-filled pastries, butter, ham, cheeses, sausages, canned meat, salads, cooked meals and sandwich fillings (Loir *et al*., 2003). High temperature usually kills *S. aureus* but not its enterotoxins which are enterotoxins A, B, C1, C2, C3, D and E (Reiser *et al*., 1984). Food is considered risky when *S. aureus* exceed 10^2 cfu/g (Little *et al*., 2001).

2.2.4 *Clostridium perfringens*

Clostridium perfringens is a Gram-positive, spore forming, rod shaped bacteria and grow under anaerobic condition (Carman *et al*., 2008). They are considered the most common microorganism due to its ubiquitous spores in soil and gastrointestinal tract of human and animals. It has the ability to grow fast and generate more than 15 toxins to cause various types of diseases. *C. perfringens* can be classified into five groups which are A, B, C, D, and E depending on the production of alpha (α), beta (β), epsilon (ε), and iota (ι) toxins (Milach *et al*., 2012).

Food poisoning caused by *C. perfringens* is common worldwide (Lindström *et al*., 2011). This is due to the heat labile enterotoxin that is produced by this microorganism. Heating the food might only kill the vegetative cells but not the spores. The spores then will germinate and produce the enterotoxin in the intestinal tract, thus, result in abdominal pain, nausea, vomiting and diarrhoea (Brynestad and Granum, 2002). It has been recorded that the unsafe number of *C. perfringens* in a food sample is more than 10^5 cfu/g which at this stage, the bacteria sporulate and produce enterotoxin (Eriksen *et al*., 2010).

2.2.5 *Vibrio cholerae*

V. cholerae is a Gram-negative bacterium (Dharmasena *et al*., 2009) which cause diarrheal diseases and can be lethal especially among patients in developing countries. The occurrence is due to the lack of clean water supply (Jain *et al*., 2010) and from previously infected person (Mandal *et al*., 2011). Faecal contamination and runoff from a source can provide nutrient for this microorganism, thus enhance their number and increase the risk for the community (Osei *et al*., 2010). A minimum number of cells required to cause illness is 10^3 (Schmid-Hempel and Frank, 2007).

The infected person will usually experience watery diarrhea with 'rice water' appearance (Dharmasena *et al*, 2009). The illness is usually treated with oral rehydration therapy and in severe cases, antibiotic will be used to shorten the duration of the disease (Mandal *et al*., 2011) and the outbreak is controlled through sewage and water treatment (Thompson *et al*., 2011). There are about 200 serogroups of *V. cholerae* but only groups O1 and O139 have been demonstrated to cause foodborne disease outbreak (Dharmasena *et al*, 2009).

2.2.6 *Campyobacter jejuni*

C. jejuni is a Gram-negative, spiral shaped bacterium. This bacterium naturally colonized birds intestine and human usually get infected from chicken (Dasti *et al*., 2010). In some cases, campylobacteriosis can also be transmitted through wild birds, sheep, pet animals and contaminated water (Wilson *et al*., 2008). An infected person will experience fever, vomiting and headache for 1 to 3 days and followed by of watery or bloody diarrhoea and abdominal pain for 3 to 7 days. Extreme cases of dehydration might cause the patient to be hospitalized (Garcia Rodriguez *et al*., 2006).

Because of the colonization of this bacterium in the animal intestine, improper handling during food preparation might allow contamination to other body parts or other foods items in the kitchen. Cooking temperature also plays an important role to ensure all bacterial cells are killed and the food is safe for consumption. Gastroenteritis is triggered by several virulence factors like resistance to bile salts, invasion of the epithelial cells and the production of cytolethal distending toxin (CDT) (Gargiulo *et al*., 2011). Infective dose of *C. jejuni* is very small which is about 500 cells (Waage *et al*., 1999).

Campylobacter jejuni were previously found to be unable to grow outside a host. However, recent research has discovered that this bacterium can adapt to environmental stress like antimicrobials, temperature and dehydration by mutation (Newell *et al*., 2010). The wide usage of drugs like fluoroquinolones (FQs) that are commonly used in human and poultry to treat infections can also render this microorganism to become resistant to the drugs (Wang *et al*., 2010). Therefore, treatment using these drugs would only worsen the condition, and require a higher dose or the introduction of new type medicine.

2.2.7 *Listeria monocytogenes*

L. monocytogenes is a Gram-positive, rod-shaped bacterium (Chen *et al*., 2010). Food items such as milk and dairy products, meat and meat products, plant products and fish and fishery products (Rivoal *et al*., 2013) are usually associated with *L. monocytogenes* contamination. Listeriosis cases caused by this bacterium is low but accompanied with high mortality rate (Gebretsadik *et al*., 2011) and the minimum number of cells for illness to occur is more than 10^3 (Lawley 2013).

Listeria monocytogenes was previously unrecognized as a significant contributor to foodborne diseases. This bacterium can grow at low temperature which makes storing food at the refrigerator can still be a risk for contamination. In addition, *L. monocytogenes* is able to adapt to extreme condition like high salinity and low pH (Ells *et al*., 2009). Research also has found out that listeriosis can lead to meningitis, cerebromeningitis, bacteremia or septicaemia which lead to death especially among immunocompromised person like HIV/AIDS patients, pregnant women, the elderly and infant (Rivoal *et al*., 2013). Another research in European countries shows and increase cases among patients aged 60 years and above (Denny and McLauchlin, 2008). This shows that although medical technology has advanced the harmful bacteria evolved and become stronger.

2.3 Factor of foodborne diseases: Agents of illness

2.3.1 Interaction between microorganisms

Most common interactions between a mixed culture of microorganisms are competitive/antagonistic and cooperative/synergistic (Yang *et al*., 2011; Castonguay *et al*., 2006 and Liu *et al*., 2006). Bacteria can secrete bacteriocins, biosurfactants, polysaccharide or enzymes like proteases or nucleases (Giaouris *et al*., 2013). Interaction occurs in several situations, for instance, fermentation, stages of foodborne illness, as well as fighting off pathogens.

2.3.2 Interaction during food fermentation

There is few cascade of interaction between several types of bacteria. These can be seen especially in the process of food fermentation. An example of the bacterial relationship is for the production of fermented soybean (black bean sauce, natto, tempeh). The enzymes produced by the microorganisms will degrade protein, fat and carbohydrate into polypeptides, fatty acids and oligopeptides which improve nutrition utilization of soybean (Zhang *et al*., 2014). The proliferation of *Bifidobacterium* can reduce the growth of harmful bacteria and improve the quality of the fermented product (Han *et al*., 2005). In addition, *Bacillus subtilis* enhanced phenolic and flavonoid content as well as the antioxidant activity of the fermented soybean (Juan and Chou, 2010).

Other types of fermented food used the symbiosis interaction of bacteria, yeasts and molds. For example, in sake brewing, the increase in nitrite levels due to the growth of nitrate reducing bacteria as well as the increase in lactic acid levels from the growth of *Leuconostoc mesenteroides* and *Lactobacillus sakei* kill wild yeast and provide a suitable condition for sake-brewing yeast. In addition, *L. plantarum* has been reported to co-aggregate with yeast for the brewing process (Furukawa *et al*., 2013).

The process of producing cheese shows a lot of microbial interactions. For instance, proteolytic activity of starter culture gives benefit to pediococci and lactobacilli in Cheddar cheese and propionibacteria in Swiss-type cheese. The flavour of the cheese also depends on the activity of these microorganisms (Peláez and Requena, 2005).

2.3.3 Production of bacteriocins

Bacteria produced secondary metabolites to interact with other strains of bacteria. For example, *S. aureus* produced its own bacteriocin, like aureocin A70 that is active against Gram-positive bacteria including *L. monocytogenes* (Coelho *et al*., 2013). Aureocin A70 is encoded within a mobilisable 8kb plasmid, pRJ6 (Netz *et al*., 2001). Bacteriocins were also found to be produced by *B. cereus.* It is active against both Gram-positive and Gram negative bacteria, thermostable and has wider pH tolerance. It has a low molecular weight which is between 3.5-6Kda (Senbagam *et al*., 2013). *Bacillus subtilis* secrete D-amino acids that become a signal for cell dispersion thus, avoiding the formation of biofilm. This bacterium can also secrete nonspermidine to inhibit biofilm formation of *E. coli and S. aureus* (Kolodkin-Gal *et al*., 2012).

Bacteriocin is widely produced by lactic acid bacteria. The existence of LAB in food is also considered safe for consumers (Macwana and Muriana, 2012). Apart from the abundance number of LAB, the bacteriocins produced by these bacteria have their own classification. They can be classified into lantibiotics (Class I), small, heat stable, cationic, amphiphilic and hydrophobic peptides bacteriocins (Class II) and larger, heat labile bacteriocins (Class III) (Ghrairi *et al*., 2008; Cintas *et al*., 2001). Examples of lantibiotics are nisin and lacticin 481 from *Lactococcus. lactis* and *Streptococcus lactis* respectively (Bierbaum and Sahl, 2009). Lantibiotics are heat stable and have small molecular mass which is less than 5 kDa (Zacharof and Lovitt, 2012). This bacteriocin binds to lipid II, a cell wall precursor thus, inhibits cell wall synthesis. It can also cause pores formation composed of nisin and lipid II molecules. Strains of bacteria that produce lantibiotics have immunity to protect themselves by the production of proteins ABC transporter, NisFEG and membrane-anchored protein, Nisl (Alkhatib *et al*., 2012).

Class II bacteriocins is a heat stable, narrow spectrum bacteriocin with less than 10 kDa of molecular weight. There are four sub-classes of class II bacteriocins which are IIa, IIb, IIc and IId (Messaoudi *et al*., 2013). Class II bacteriocin enter cytoplasmic membrane of the target cell and cause membrane depolarization and cell death (Balciunas *et al*., 2013). Class IIa bacteriocins

have been reported to be used widely due to their large number, activities and potential applications (Ennahar *et al*., 2000). Examples of class II bacteriocins are lacticin F and lactococcin G (Zacharof and Lovitt, 2012). Class III bacteriocins have a large molecular weight which is more than 30kDa. They promote lysis of the cell wall of target microorganism (Balciunas *et al*., 2013).

Lactic acid bacteria like *Lactococcus lactis, Lactobacillus fermentum, L. delbrueckii, L. plantarum, L. paracasei and Enterococcus faecium* (Sakamoto *et al*., 1998; Hugenholtz and Kleerebezem, 1999; Landeta *et al*., 2013) are usually associated with fermented food. However, they too are able to dominate microflora of many food items. This is due to the fact that, they are abundance in the environment and compete for nutrients and at the same time produce antimicrobial active metabolites like lactic acid and acetic acid, hydrogen peroxide, and bacteriocins (Ghanbari *et al*., 2013). These substances are important to ensure the survival of the microorganisms themselves and to preserve the food that becomes the medium of their growth.

Lactic acid bacteria like *Lactobacillus plantarum* were found to produce antibacterial against a wide range of foodborne and spoilage bacteria. The substance was also found to be heat stable (Suma *et al*., 1998). Several types of bacteriocins from *L. plantarum* are plantaracins B, SIK-83, A, BN, S&T, LC74, SA6, KW3O, LP84 and MG (Suma *et al*, 1998, Sand *et al*., 2010, Man *et al*., 2012). Some of the bacteriocins are active against *Enterobacter*, *Enterococcus*, *Lactobacillus*, *Pseudomonas*, *Streptococcus* and *Staphylococcus* and different serotypes of *Listeria* spp (Todorov *et al*., 2011). The protective effect of LAB is not limited to bacteria only, but also towards fungi like *Fusarium culmorum, Penicillium chrysogenum, P. expansum, Aspergillus fumigates, A. Versicolor, A. niger, Debaryomyces hansenii and Candida parapsilosis* (Cizeikiene *et al*., 2013).

A study by Cálix-Lara *et al*. (2014) discovered the ability of commercial LAB to reduce the number of *Escherichia coli* O157:H7 and *Salmonella enterica* on spinach leaf surfaces. The study also discovered the production of L-lactic acid and bacteriocin-like inhibitory substance (BLIS). The experiment mimicked a real life situation where vegetables like spinach were usually contaminated during post-harvest session. Trias *et al*. (2008) found the ability of LAB to reduce the number of pathogenic bacteria on apple and lettuce without cause tissue damage on these RTE foods. Jones *et al*. (2008) discovered that LAB can be protective cultures in vacuum-packed chill-stored meat because they can reduce the number of spoilage microorganisms. Other types of food that can be colonized by LAB are seafood (Ghanbari *et al*., 2013), cereal (Oliveira *et al*., 2014, Coda *et al*., 2014) and sausages (Landeta *et al*., 2013).

2.3.4 Production of biosurfactants

The growth of FBB are sometimes controlled by the production of biosurfactants or bioemulsifiers. Examples of biosurfactants are rhamnolipids from *Pseudomonas aeruginosa,* surfactin from *Bacillus subtilis,* emulsan from *Acinetobacter calcoaceticus* and sophorolipids from *Candida bombicola*

(Nitschke and Costa, 2007). A rhamnolipid mixture was shown to inhibit the growth of *E. coli, micrococcus luteus, Alcaligens faecalis, Serratia marcescens, Mycobacterium phlei and S. epidermidis* (Abalos *et al* 2001). Studies by de Araujo *et al*. (2011) and Magalhães and Nitschke (2013) showed the ability of rhamnolipid and surfactin to inhibit *L. monocytogens.* Several studies have shown the ability of biosurfactants to prevent biofilm formation by *Salmonella enterica, E. coli, Proteus mirabilis* and *L. monocytogenes* (Simões *et al*., 2010).

2.3.5 Quorum sensing

Bacteria can also communicate among themselves. This action is called quorum sensing (QS) which involves the creation of signalling molecules called autoinducers (Dourou *et al*., 2011). Bacteria will regulate gene expression to ensure their survival after receiving signals about critical level of molecules (Federle and Bassler, 2003). *Salmonella* does not possess a luxl gene that codes for acylated homoserine lactones (AHLs), a component crucial for QS. However it has the gene homolog known as *SdiA that* is used for detecting signals from other species of microorganism (Dourou *et al*., 2011). *E. coli* O157:H7 is one of the bacteria that produce autoinducer-2 (AI-2), that signals for the formation of biofilm, chemotaxis, flagellar synthesis and motility (Pillai and Jesudhasan, 2006). Therefore, the production of biofilm and AI-2 can cause a difficulty to control cross contamination (Silagyi *et al*., 2009). *Pseudomonas aeruginosa* choose to stay dormant and perform only after their population overcome the host's defence mechanism and the signals involve in this operations are oligopeptides (5-10 amino acid cyclic thiolactane), N-acyl homoserine lactones (AHLs), furanosyl borate (autoinducer-2), hydroxyl-palmitic acid methylester and methyl dodecanoic acid (Kalia, 2013).

In *V. Cholera,* QS is the most important part for virulence gene expression. The formation of biofilm enables *V. cholerae* to become acid resistance and can easily enter the stomach. In the intestine, genes expression for *vps* (Vibrio polysaccharide synthesis), *ctx* (cholera toxin) *and tcp* (toxin-co-regulated pilus) are repressed. The bacterium detaches from the biofilm and colonizes the intestines. Here, the signals are low, thus, cholera toxin and toxin coregulated pilus are expressed. After the population increased, quorum-sensing signals rise, virulence genes are repressed and haemagglutination (HA) protease is produced leading to detachment from the epithelium and exiting of the host (Milton, 2006). Quorum sensing is also important in *L. monocytogenes,* whereby the AI-2 molecules and *luxS* gene that function its precursor, involved in the regulation of the biofilm (Bonsaglia *et al*., 2014). In this research they also discovered that *L. monocytogenes* stick on stainless steel and glass surfaces better and form biofilm effectively at 35 °C after 48 h.

2.4 Factor of foodborne diseases: Environment

2.4.1 Relationship of foodborne illness with hygiene level of restaurants

There is little information relating grades of food premises with foodborne diseases. Generally, premises with unacceptable food hygiene level are indications that eating at the premises will increase the risk of foodborne diseases (Djekic *et al*., 2014 and Lee *et al*., 2012). However, based on a research by Simonne *et al*. (2004), the incidence of illness was reported to be much higher due to the consumption of Mexican cuisine that scores more in premise cleanliness compared to consuming Asian cuisine that has a lower score. In another case, a Danish restaurant which serves the cheapest set menu at £174 was found to cause food poisoning to 67 to 78 customers for five days. Norovirus was suspected to be the agent of transmission (Alsop, 2013). Sometimes, in a well-known and busy restaurant, waiters provide used fork which is cleaned with soiled linen to the customers due to lack of time to meet all of the customers' requirements (Walczak and Reuter, 2004).

2.5 Factor of foodborne diseases: Host

2.5.1 Consumers' tolerance towards foodborne illness

People who eat ethnic food which they are not familiar with can easily get sick. Gormley *et al*. (2012) reported that in England and Wales, ethnic food like Chinese, Indian and Italian caused outbreak from 1992 to 2009. The agent for food poisoning cases was also found to be specific, for instance, *Salmonella* spp. for Chinese cuisine, *Bacillus* spp. for Indian cuisine and *S. Enteritidis* PT4 for Italian cuisine. Bacteria for foodborne illness has been reported to be ethnic and racial specific as well; for instance, the association of *Campylobacter, Listeria, Shigella* and *Salmonella* infection among Hispanics, *Shigella* and *Yersinia* infection among African American, *Yersinia, Vibrio* and *S. typhi* infection among Asians (Gerald and Perkin., 2003).

Traveller's diarrhea is a type of foodborne illness affecting travellers and can cause significant morbidity among travellers that visited developing countries (Diemert, 2006). Travellers get diarrhea by eating or drinking what is commonly consumed by local residents. Local people are not affected due to the development of immune system after constant exposure to the pathogens and the presence of good bacteria that protect the gut. The exclusion of bad bacteria is strain specific (Gueimonde *et al*., 2006), suggesting the reason why certain people had a low tolerance for foreign food. Commonly, acute diarrhea will resolve in two weeks; but longer than this period, it is considered as chronic diarrhea (Ardley and Wright, 2010). Bacteria such as *Escherichia coli, Salmonella spp., Shigella* spp*. and Campylobacter* spp. are usually associated with traveller's diarrhea (McGregor *et al*., 2012).

2.5.2 Human digestive system and its protective features

The incidence of foodborne illness among a different background of people is related to their tolerance to foodborne pathogens. The human digestive system contains more than 300 species of bacteria that work together for the benefit of the host. The examples of their advantages are the supply of energy from fermentation, synthesizing vitamin B and K, and metabolism of bile acids sterols and xenobiotics (Cummings and Macfarlane, 1997). Normal flora of human gastrointestinal tract is mostly *Bacteroides and Bifidobacterium.* In addition to that *Helicobacter pylori;* which is known to cause gastric ulcers; lactobacilli, *Enterococcus faecalis, E. coli,* coliforms, enterococci, clostridia and lactobacilli are also part of human GI tract. The presence of anaerobic methanogens can also be found in human colon (Todar, 2012).

Normal flora is not digested because they are recognized as part of the digestive system, therefore immune system does not harm them. In addition, *Bacteroides* species have a special coating that protects them from the immune system (Senior, 2013). Gut microflora can also train human immune system and prevent the growth of pathogenic bacteria (Crost *et al*., 2010), like probiotics, which are known to aid in the prevention of diseases (Guarner and Malagelada, 2003). Furthermore, constant interaction between host and foreign bacteria can benefits human (Salminen *et al*., 1998). This might be the reason why carriers of a pathogenic microorganism cause illness to other persons but not themselves; and why people from different places/countries have different tolerance towards unclean food.

2.5.3 Bacterial interaction in the intestine

The protection of human gut is usually associated with probiotics. There are several levels of host-microbe interaction. Firstly, microbe-epithelium interface which includes adhesion to mucosal and epithelial cells and production of mucus secretion, thus protecting the gut; secondly, microbe-immune system interaction, which consists of immune response in the gut system; and finally, microbe-microbe interaction, which comprise of the exclusion and inhibition of pathogens through prevention of their adhesion and replication. This is done by secretion of antimicrobial agents, nutrients competition and anti-toxin effects (Collado *et al*., 2010).

A study by Ingrassia *et al*. (2005) discovered that the adhesion of *Lactobacillus casei* to the epithelial cells inhibits the invasion of *E. coli* and this reaction is not due to the bactericidal effect of *L. casei.* They came out with a theory that the adhesions of *L. casei* secrets compounds that induce their adhesion thus decrease *E.coli* growth due to the competition for attachment site. A similar result was observed by Gopal *et al*. (2001) that found out the antagonistic activity of *Lactobacillus rhamnosus, L. acidophilus* and *Bifidobacterium lactis* against *E. coli* O157:H7. The adherence of these bacteria hampers the colonisation of this pathogenic *E. coli* O157:H7. Another study by Carey *et al*. (2008) found out that the regulation of gene *stx2A* for Shiga toxin production

may be related to the changes in pH due to the production of organic acid by probiotics bacteria.

Bacterial interaction can also cause genetic recombination where they transfer genes that create new types of bacteria that might become resistant to antimicrobial agents. This process occurs through conjugation, transformation and transduction (Wilkins and Frost, 2001). For example, the emergence of *E. coli* O157:H7 which was originated from *E.coli* O55:H7 that caused diarrhea in infants (Kelly *et al*., 2009); pathogenic bacteria that receive genetic elements from probiotics, thus increase their ability to colonize human gut (Capozzi and Spano *et al*., 2009); and *Enterococcus* spp that are able to transfer antibiotic resistance genes to its own species as well as to *S. aureus* and *Listeria* spp. This bacterium can also cause a mutation to non-pathogenic bacteria in the gut, environment and food (Pesavento *et al*., 2014). In addition, the extensive use of antibiotic for growth promotion in an animal can also increase the number or resistant bacteria in food and human being.

2.6 Antimicrobial resistance of foodborne bacteria

The wide use of antibiotics has rendered foodborne bacteria resistant to certain types of antibiotic. These antibiotics were firstly used to kill microorganisms that might cause infection in the animal as well as to encourage animal growth (White *et al*., 2002). However, these bacteria tend to evolve and became stronger, hence difficult to be killed. This required the usage of another type of antibiotics which has a better effectiveness, but the cycle might start again, thus the fight against antimicrobial resistance microorganisms will become more complicated.

Some bacteria are multi-resistance, for instance, *Salmonella typhimurium* which is resistance to nalidixic acid and ciprofloxin (Newell *et al*., 2008). Other antimicrobial species of *Salmonella* like *S. typhi* and *paratyphi A* has been shown as a cause of mortality in developing countries (Mermin *et al*., 1999). In a study conducted by Thong and Modarressi (2011), *Salmonella* were isolated from retail meats and street foods. From 88 strains, the highest resistance was to tetracycline, followed by sulfonamide, streptomycin, nalidixic acid, trimethoprim–sulfamethoxazole, ampicillin and chloramphenicol. Less than 10% were resistant to cephalotin, kanamycin, ciprofloxacin, gentamycin, cefoxitin, amoxicillin–clavulanate and amikacin. In addition, 67% of the isolates were multidrug resistant. They also found that the strains possessed ten out of 17 resistance genes which are blaTEM-1, strA, strB, aadA, sulI, sulII, tetA, tetB, floR, cmlA.

Methicillin-resistant or Oxacillin-resistant *Staphylococcus aureus* (MRSA) is another strain that resistance to methicillin antibiotic. MRSA is usually associated with a nosocomial or hospital-acquired infection which can cause infection in the respiratory tract, urinary tract and open wounds. Nevertheless, MRSA has been found in various animals like cattle, chicken and pigs. A study by de Boer *et al*. (2009) realized that MRSA contaminates 11.9% of raw meat indicating that livestock could be a source of serious infection, especially

among patients through contamination from food handlers. In another research, MRSA was found in raw milk and dairy products in Egypt (Kamal *et al*., 2013), suggesting that improper food handling can easily cause the spread of this microorganism into the community. Hanson *et al*. (2011) found low prevalence (1.5%) of MRSA isolated from pork meat in Iowa indicating the spread of this bacterium into meat producing animals has become a significant issue and require attention from the authorities.

Some *E. coli* is known as a normal flora in the human digestive system. However, through mutation, this commensal *E. coli* can become pathogenic. In a study by Zhao *et al*. (2011), 57 *E. coli* strains were isolated from bovine endometritis, which is an infection of the inner lining of the uterus. The study found out that all isolates were resistant to sulfamethoxydiazine and trimethoprim whereas, most of the strains were resistant to sulfadiazine, tetracycline, oxytetracycline, cefazolin, and chloramphenicol. In addition, 80.7% of the isolates exhibit multiresistant which are resistant to more than 10 antibiotics. In a different study the transfer of resistant genes from *E. coli* to *Salmonella* is found to be achievable (Poppe *et al*., 2005). This proves that intestinal tract of animal can become a perfect medium for a bacterium to turn other bacterium resistance to antimicrobial agents. The exchange of resistant genes between clones of bacteria has also been shown in water, soil and kitchen utensils (Walsh *et al*., 2008).

In a study by Yan *et al*. (2010), antimicrobial resistance among *Listeria monocytogenes* was observed for ciprofloxacin, tetracycline and streptomycin. Almost 20% of the overall strain tested was found to have multiple resistance properties. Some of the strain carried and tet(S) and tet(M) gene which promotes resistance to tetracycline (Burdett, 1996). They also have Tn916 gene which allows for the conjugative transfer of resistance gene to other cells thus make the neighbouring cells resistance to antibiotic as well (Roberts and Mullany, 2011). Conter *et al*. (2009) found out that *L. monocytogenes* are normally resistance to one type of antibiotic, commonly associated with clindamycin. Other types of antibiotics that have no effect on *L. monocytogenes* are linezolid, ciprofloxacin, ampicillin, rifampicin, trimethoprim/ sulphamethoxazole, vancomycin and tetracycline. These antibiotics are usually used in veterinary and treatment against listeriosis.

2.7 Advanced procedure for detection of foodborne bacteria

Detection of foodborne bacteria using conventional methods was quite time consuming and laborious. Food items contain several compounds like protein, carbohydrates and oils which have an effect on the isolation and analysis (Swaminathan and Feng, 1994). The development of technologies created various ways for the detection of microorganism even in a small number of the cell. The procedures are faster and culture-independent which is specially developed for molecular studies and microbial diversities (Su *et al*., 2012)

2.7.1 Traditional methods

The isolation of FBB is also affected by the number of normal flora existed in the food. The presence of these bacteria interfere the process especially when the number of FBB is low (DuPont *et al*., 1989). To overcome this problem, the food sample is transferred into an enrichment medium to increase the growth of the desired bacteria and suppressed the normal microflora. Then the samples will be cultured on selective media to presumptively detect the colony of the bacteria macroscopically. Several biochemical tests are used for further identification and serotyping can proceed if necessary. Even though the conventional method is time-consuming, this method of bacterial identification is still effective and accurate (Velusamy *et al*., 2010).

2.7.2 Biochemical test kits

The traditional method, however, has a weakness like failing to identify certain types of microorganisms because the domination of culturable microorganisms (Justé *et al*., 2008). Over the years, rapid identification methods have been developed to provide a faster result. Some of the techniques include miniaturized biochemical kits, antibody-based tests, and assays which can speed up analysis. Miniaturized biochemical kits like API® strip are small and easily handled in the laboratory. After inoculation and incubation, the changes of the media colour can be compared with the databases result in the company's website, thus provide us with the species name. Other examples of biochemical test kits are Cobas IDA, RapID and Vitek (Feng, 2001).

2.7.3 Liquid chromatography

Another advance technique is developed to detect the toxins that are produced by certain microorganisms. Basically, liquid chromatography is used to separate a mixture of the compound in order to identify a component in the mixture. A study by Sospedra *et al*. (2012) used liquid chromatography to identify staphylococcal enterotoxin B produced by *S. aureus* in milk. In this experiment, they found out that the levels of enterotoxin B in the milk sample was 3.6 µg/mL. The cereulide toxin, produced by *Bacillus cereus* was also identified using liquid chromatography-tandem mass spectrometry (LC-MS/MS) with the range of 0.1 – 0.5 ng/mL (Yamaguchi *et al*., 2013).

2.7.4 Enzyme-linked immunosorbent assay (ELISA)

Enzyme-linked immunosorbent assay (ELISA) which uses the concept of one antibody is specific for one antigen. A study by Zhong *et al*. (2012), used sandwich ELISA to detect *Vibrio parahaemolyticus* using anti- *V. parahaemolyticus* IgY as capture antibody and rabbit anti- *V. parahaemolyticus* IgG as the detection antibody. The optical density (OD) was read using ELISA reader.

2.7.5 Polymerase chain reaction (PCR)

The molecular method has become preferable due to a faster, specific and more accurate result and able to justify the diversity of microorganisms in the environment (Justé *et al*., 2008). In a research conducted by Naravaneni and Jamil (2005), *Salmonella* and *E. coli* were detected using Polymerase Chain Reaction (PCR) system. Firstly the specific primers for these bacteria were designed and the reaction mixtures were placed in the thermal cycler. After completion, the agarose gel can be viewed under UV transilluminator. Only specific DNA with a specific length from the bacteria can be viewed as a single line on the agarose gel indicating the existence of the pathogen. Quantitative PCR, a new approach on bacteria detection based on conventional PCR is used to detect bacteria that cause enterotoxaemia in the animal. A research conducted by Albini *et al*. (2008) detected toxin released by *Clostridium perfringens* via this assay. Shiga toxin producing *E. coli* were also detected in minced beef and dairy products using quantitative PCR (Derzelle *et al*., 2011).

2.7.6 Pulsed-field gel electrophoresis (PFGE)

Pulsed-Field Gel Electrophoresis (PFGE) is one of the most popular methods used in foodborne research. The purpose of PFGE is to establish a database for genotyping of the microorganism. This technique will eventually produce a dendogram or phylogenetic tree to show the relatedness of different strains of one microorganism. In a research conducted by Peles *et al*. (2007), milk samples were collected from 20 farms for the detection of *S. aureus.* From the PFGE pattern, they found out that there were 59 strains of *S. aureus.* A dendogram is also used to show us about the diversity of each strain of bacterium.

2.7.7 Denaturing gradient gel electrophoresis and temperature gradient gel electrophoresis (DGGE/TGGE)

The main advantages of using DGGE and TGGE are due to their affordability for molecular laboratories and the simplicity of results interpretation. The bands can be easily identified through sequencing (Justé *et al*., 2008). DGGE analysis consists of several procedures such as extraction of DNA from a sample, amplification of the DNA with specific primers, separation of amplicons based on sequence differences and finally the analysis of obtained patterns (Balázs *et al*., 2013). During electrophoresis process, a denaturing agent like formamide and urea are used to separate the DNA molecules. This process is done at a constant heat around 60°C.

DGGE technique separate DNA based on electrical charge, shape and molecular weight. During the denaturation process, the separation process will cause the molecule to slow down. DNA with the same melting domain will be separated depending on the nucleotides pairs, which are adenine (A) with thymine (T) and cytosine (C) with guanine (G). The melting temperatures and

increasing gradient of the denaturing chemical will separate them at a different position on the gel. Thus, high GC content requires high concentration of denaturing chemical to melt before migration further down the gel (Muyzer *et al*., 1993). In TGGE, during the migration of the DNA molecules, the temperature is increased, therefore, the strands will separate which cause the movement to decrease. The movement also depends on the GC content of DNA molecules (Muyzer and Smalla, 1998).

2.7.8 Microarray

DNA microarray consists of DNA probes that are bound to the solid substrate. Each spot is measured around 50 to 150 μmeter and is composed of many probes that complement with the gene of interest. During the hybridization process, the target DNA will diffuse across the glass surface and complementary sequences will anneal to a probe and form DNA duplex. The targets can be detected using several reporter molecule systems. The principle used in microarray is quite similar with other hybridization and detection techniques like Southern and Northern blotting (Call, 2001).

A lot of studies used DNA microarray technique to detect foodborne pathogen immediately. Kim *et al*. (2008) found out that the microarray can be used to discriminate non-pathogenic and pathogenic microorganism. This is because food industry use some good bacteria as well to produce fermented food and cultured milk drink. Therefore, the ability to differentiate between those bacteria is important. Bang *et al*. (2013) and Suo *et al*. (2010) also discovered the ability of microarray method to distinguish between very low amounts of FBB. Thus, the study is very suitable to be used in the food industry due to its rapidity as well as accuracy.

2.7.9 Pyrosequencing

Pyrosequencing is an advanced method to generate DNA sequence of microorganism. The steps involved in pyrosequencing are the hybridization of single stranded DNA with the aid of four types of enzymes such as DNA polymerase, ATP sulfurylase, luciferase and apyrase. The addition of substrates leads to several reactions and produce visible light that represents the number of nucleotides incorporated into the DNA. As the process of nucleic acid polymerization occurs, inorganic pyrophosphate (PPi) is released. Then, the released of PPi will be converted to ATP by sulfurylase and finally will be sensed by luciferase to produce light. A nucleotide that is not incorporated will be enzymatically removed to continue the reaction with newly added nucleotide (Langaee and Ronaghi, 2005).

Pyrosequencing has many significant applications such as genotyping of single nucleotide polymorphisms (SNP), resequencing, taq sequencing, analysis of difficult secondary structures, microbial and viral typing, as well as fungal and bacterial identification (Fakruddin *et al*., 2012). The purpose of genotyping of SNP will determine the variation occurred in a nucleotide between species or

chromosome. In addition, SNP can help to study how human interact with pathogens, drugs, vaccine and chemicals (Carlson, 2008).

Resequencing of gene that has been associated with the disease can help to quantify and determine mutations because of the accuracy of this technique whereas; tag sequencing will determine the length of a DNA and gene identification (Fakruddin *et al*., 2012). In an analysis of difficult secondary structure like hairpin RNA, pyrosequencing is able to solve the problem and determine its sequence (Ronaghi *et al*., 1999) for the study of gene transcription and replication (Fakruddin *et al*., 2012). Microbial and viral typing are also important for identification. This will lead to the production of appropriate treatment, therapies and drugs. Identification of fungal and bacterial can give a lot of information about the species and to group them correctly according to the result of pyrosequencing.

CHAPTER 3

MATERIALS AND METHODS

3.1 Study location

The study location was in Seri Kembangan, Selangor, Malaysia.

3.2 Duration of the study

The study was conducted from October 2012 to August 2015.

3.3 Sampling unit

The primary sampling unit in this study was cutting boards (CB) collected from various food premises in Seri Kembangan, Malaysia. In this study 26 CB were taken for analysis. Cutting board was chosen because it is one of the most used kitchen utensils and has contact with various food items. Some cutting boards are too porous which makes them harder to be properly cleaned and become an ideal place to grow bacteria. This is why CB became a good candidate for this study instead of other kitchen utensils.

3.4 Variables

Table 3.1 Independent and dependent variables in the study

Independent variables	**Dependent variables**
Bacterial diversity	Premise grades
Total number of bacteria	Premise grades, types, usages, and conditions of cutting board (CB).
Foodborne pathogenic bacteria	Premise grades, types, usages and conditions of CB.
	Different phyla, class, family, genus of bacteria
	High and low number of background bacteria

3.5 Instruments

The research instruments used in this study were wet sterile sponges (3.8 x 7.6 cm), sterile stomacher bag, sterile buffered peptone water, homogenizer, trypticase soy agar, DNA isolation kit, DNA purification kit, vortex mixer, NanoDrop spectrophotometer, thermal cycler, pyrosequencing platform and Real-Time PCR System.

3.6 Sample collection

In this study, 26 cutting boards were obtained between July and August 2013. These premises sell typical Malaysian food like rice, vegetables, chicken, beef, cut fruits and sea-food using self-service selling method where customers take their dishes by self-selection and pay at the cashier.

The grades of the premises where the CB samples are taken were identified. Grades A, B, and C reflected the level of cleanliness of the premises, which represent clean, moderately clean and not clean, respectively (Table 3.2). Information of the CB was also recorded. Types of cutting boards were divided into plastic and wood and usages represent the food materials being cut on the CB like common (for all types of foods), fruits, vegetables and chicken/beef.

Conditions of the CB were divided into average, clean and dirty. This was done through observation of the physical condition of the CB surfaces through naked eyes. “Clean” means no traces of food materials; “average” means small traces of food materials, whereas “dirty” means traces of old stain with the presence of some food materials.

To confirm the presence of live bacteria cells on the CB, 100 µl of samples were taken and grown on trypticase soy agar (TSA) using spread plate technique. The results showed that all samples contain a high number of bacteria, which we recorded as too numerous to count (TNTC).

In addition, CBs collected were randomly given by the food handlers and did not undergo any special treatment prior to the collection. The length of usage was beyond the familiarity of the food handlers. Some of the CBs were given as samples because they were not in used during the visit. In addition, during the collection, CBs were basically unwashed or washed using detergent and water only.

Table 3.2 Samples of cutting board taken from different grades of food premises and their types, usages and conditions

Samples	Premise grades	Cutting board (CB)		
		Types	Usages	Physical conditions
cb1	A	Plastic	Common	Dirty
cb2	A	Plastic	Common	Clean
cb3	B	Plastic	Common	Dirty
cb5	B	Wood	Common	Dirty
cb6	B	Wood	Common	Clean
cb7	A	Plastic	Vegetables	Clean
cb8	NG[a]	Plastic	Fruits	Clean
cb9	B	Plastic	Vegetables	Clean
cb10	C	Plastic	Fruits	Clean
cb11	C	Plastic	Vegetables	Dirty
cb12	B	Plastic	Common	Dirty
cb13	B	Wood	Vegetables	Clean
cb14	B	Plastic	Vegetables	Average
cb15	B	Wood	Vegetables	Dirty
cb16	B	Plastic	Beef	Clean
cb17	B	Wood	Vegetables	Clean
cb18	B	Wood	Vegetables	Clean
cb19	B	Wood	Beef	Average
cb20	B	Wood	Vegetables	Clean
cb21	B	Wood	Common	Average
cb22	B	Wood	Chicken	Clean
cb23	A	Plastic	Vegetables	Clean
cb24	B	Wood	Chicken	Clean
cb25	C	Wood	Vegetables	Clean
cb26	A	Wood	Vegetables	Clean
cb27	B	Wood	Beef	Average

[a] No grade, subsequently grouped with C for analysis.

3.7 Bacterial DNA extraction from cutting board

Bacterial DNA was obtained from the cutting boards according to the methods of Gómez et al. (2012) with some modification. Basically, wet sterile sponges 3.8 x 7.6 cm (3M^{TM}, USA) were used to swab approximately 10cm^2 area on the center of the cutting boards using 40-vertical S-strokes. The sponges were then immersed in 90 mL of sterile buffered peptone water (Difco, USA) and homogenized for 10 min in a sterile stomacher bag (Interscience, France). The

extraction of bacterial DNA was done directly without culturing. A total of 1.8 mL of the sample was taken for DNA extraction using UltraClean Microbial DNA Isolation Kit (MO Bio Laboratories, USA) according to manufacturer's instruction.

Basically, 1.8 mL of culture was added to 2 mL collection tube. The tube was centrifuged at 10 000 × g for 30 s. The supernatant was decanted and the tube was centrifuged again at 10 000 × g for 30 s. The supernatant was removed with a pipette tip. 300 µL of microbead solution was added to the tube and vortexed to mix the solution. The solution was transferred into microbead tube and 50 µL of solution MD1 was added. The microbead tube was vortexed horizontally for 10 min at maximum speed. Then, the tube was centrifuged at 10 000 × g for 30 s. The solution was transferred to 2 mL collection tube and 100 µL of solution MD 2 was added to the tube. The solution was vortexed for 5 s then incubated at 4 ºC for 5 min. The tube was centrifuged 10 000 × g for 1 min and transferred to 2 mL collection tube, avoiding the pellet. Solution MD3 was initially shaken and 900 µL was added to the collection tube and vortexed for 5 s. 700 µL of the solution was transferred to spin filter tube and centrifuged at 10 000 × g for 30 s. The remaining solution was added and the process was repeated. Then, 300 µL of solution MD4 was added into the solution and centrifuged at 10 000 × g for 30 s. The flow through was discarded and centrifuged again at 10 000 × g for 1 min. The spin filter was put on a new 2 mL collection tube and 50 µL of solution MD5 was added. The tube was centrifuged at 10 000 × g for 30 s. The flow trough contained the DNA and was stored at -20 ºC until they were used for further analysis. DNA concentration was determined using Nanodrop 2000 (Thermo Scientific, USA).

3.8 Pyrosequencing

The 16S rRNA gene fragments were amplified by PCR using the primers, F357 (5'-CCTACGGGAGGCAGCAG-3',) and R926 (5'-CCGTCAATTCCTTTRAGTTT-3',) as forward and reverse primers respectively. Short tag sequences were also inserted for analysis (Table 3.3). Each PCR reaction mixture consisted of 25 µL of Premix Ex Taq (Takara Bio, Japan), 2.5 µL of forward and reverse primer (10µM) respectively, 2.0 µL of the template and 18 µL of dH_2O. The condition of the thermal cycler (Takara, Japan) was set as follows; 94 ºC for the first 5 min, followed by 30 cycles of denaturation (94 ºC, 40 s), annealing (50 ºC, 40 s) and extension (72 ºC, 1 min). Then, the temperature was set for 72 ºC for an additional 5 min and finally at 4 ºC as the holding temperature. Final PCR products were purified using QIAQuick PCR purification kit (Qiagen, Germany). There were two steps for purification. The amplified fragments were then used for the pyrosequencing analysis performed by 454 GS FLX Titanium XL+ Platform (Roche, Switzerland).

Table 3.3 Tag sequences inserted for pyrosequencing

Samples name	Tag sequences
cb1	CAGTACGTACT
cb2	CGATACTACGT
cb3	CTACTCGTAGT
cb5	ACGATGAGTGT
cb6	ACGTCTAGCAT
cb7	ACTCACACTGT
cb8	ACTCACTAGCT
cb9	ACTGATCTCGT
cb10	ACTGCTGTACT
cb11	AGACACTCACT
cb12	AGACGTGATCT
cb13	AGATACGCTGT
cb14	AGTATGCACGT
cb15	AGTCTGTCTGT
cb16	ATCGTCAGTCT
cb17	ATCTGAGACGT
cb18	ATGCTACGTCT
cb19	CACTACGATGT
cb20	CAGTCTCTAGT
cb21	CGAGACACTAT
cb22	CGTATAGTGCT
cb23	CTAGACAGACT
cb24	CTATCGACACT
cb25	CTCACGTACAT
cb26	AGTACGAGAGT
cb27	AGTAGACGTCT

3.9 Analysis of DNA sequences

Raw sequence files underwent demultiplexing, deletion of low quality (<25, Phred score ≥20) and barcoded sequence, as well as chimera detection (Huang et al., 2014). The Quantitative Insights into Microbial Ecology (QIIME) software was used for operational taxonomy units' (OTUs) picking and diversity analysis (Kakizaki et al., 2012). The sequences were then confirmed using the Ribosomal Database Project (RDP) Classifier (http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp).

3.10 Real-time quantitative PCR

The total bacterial population was determined using real-time quantitative PCR (qPCR). The production of the standard curve was using *E. coli* BW25113 which generated an equation of y = -3.711x + 54.73, with R^2 = 0.9941 and efficiency value = 86%. Templates for qPCR were reusing DNA samples previously prepared for pyrosequencing. The 16S rDNA was amplified using 340f (5'-TCCTACGGGAGGCAGCAGT-3') and 781r (5'-GGACTACCAGGGTATCTAATCCTGTT-3') as forward and reverse primers respectively and 5'-CGTATTACCGCGGCTGCTGGCAC-3' was used as TaqMan probe. Reaction mixture consists of 10 µL of TaqMan Fast Advanced Master Mix (Applied Biosystems, USA), 0.72 µL forward and reverse primer (25 µM), 0.34 µL of TaqMan probe, 6.22 µL of nuclease-free water and 2 µL of DNA template (1pg to 100 ng). The thermal profile of the Real-Time PCR System (Applied Biosystems, USA) was set as follows, 2 min at 50 ºC for the uracil-N-glycosylase incubation, 20 min at 95 ºC for polymerase activation, followed by 40 cycles of denaturation (1 s at 95 ºC), and simultaneous annealing and extension (20 s at 60 ºC).

3.11 The accuracy of analysis using 16S rRNA gene sequencing

This study would like to emphasize on the accuracy of 16S rRNA gene analysis. Although this technique has emerged as a preferred method for fast and reliable bacterial identification, the sequences in some databases are not 100% accurate, no general agreement of genus or species based on the data and might present microheterogeneity within a species (Claridge, 2004). Therefore, in this study, the bacterial names especially on the genus and species level are based on the highest percentage (>97%) to the taxonomy in the database, including the name of the strains or subspecies of bacteria like *E. coli* O157:H7.

3.12 Analysis of pyrosequencing data

Analysis of pyrosequencing data was done using software called QIIME (Quantitative Insights into Microbial Ecology). Firstly, Mapping File was created. The file contains information about the samples for the analysis of the name for each sample, the sequence used and the sequence of the primer for amplification.

The second step is to assign samples to multiplex reads based on their nucleotide barcode. This step also removed low quality or ambiguous reads. The third step, involving a chimera check which was done using Black Box Chimera Check (B2C2). This software removed any chimera reads. Chimeras are hybrid products between multiple parent sequences that can be falsely interpreted as novel organisms, thus inflating apparent diversity (Haas et al., 2011).

The fourth step was assigned for picking Operational Taxonomic Units (OTUs). Sequences with greater than 97% similarity were assigned to the same species.

The result was generated through several steps as follow:

1. Picking OTUs based on sequence similarity within the reads.
2. Picking a representative sequence set, one sequence from each OTU.
3. Aligning the representative sequence set.
4. Assigning taxonomy to the representative sequence set.
5. Filtering the alignment prior to tree building - removing positions which are all gaps, or not useful for phylogenetic inference.
6. Building a phylogenetic tree.
7. Building an OTU table.

The fifth step involved a similarity search by Ribosomal Database Project (RDP) of the OTUs sequence. Using QIIME software, alpha diversity was computed and rarefaction curves were generated.

The steps involved in alpha rarefaction plots were

1. Generate rarefied OTUs table.
2. Compute measures of alpha diversity for each rarefied OTU table.
3. Collate alpha diversity results.
4. Generate alpha rarefaction plots

The final step was to compute beta diversity and generate beta diversity plots.

The scripts performed the following steps:

1. Remove sample heterogeneity: Standardize the data obtained from samples with different sequencing efforts, and compare the OTU richness using standardize platform.
2. Compute beta diversity: Assess the differences between microbial communities.
3. Generates principal coordinates: Principal coordinate analysis (PCoA) helps to extract and visualize informative components of variation from complex, multidimensional data. It provides an intuitive visualization of the data structure.
4. Generate emperor PcoA plots: Inspection of PCoA plots in three-dimensional files.

After the process of identification finished, the file was downloaded for further analysis. The results from RDP were preferable as it covers species level as well. All of the data obtained were copied and pasted to Microsoft Excel format. Microorganisms with the same OTU were sorted and the one with the highest percentage was chosen. Then, all of the microorganisms were compiled and their total quantity was calculated. Microsoft Excel was then used to prepare heat map or graph based on the result.

3.12 Statistical analysis

A statistical analysis was done using the software IBM SPSS Statistic 22. One way ANOVA and t-test were used to determine the relationship between premise grades and bacterial richness. The analysis was also used to determine the mean difference of a total number of bacteria and the number of FBB based on premise grades, types, usage and condition of CB.

Multiple regression and correlation test was used to evaluate the relationship between FBB and different phyla, classes, orders, families and genera of bacteria. This test showed which bacteria supported or reduced the FBB growth.

CHAPTER 4: ANALYSIS OF MICROBIAL COMMUNITY AND FOODBORNE PATHOGENIC BACTERIA AND THEIR CORRELATION WITH GRADES OF FOOD PREMISES

4.1 Introduction

Foodborne disease is a condition where a person experienced diarrhoea, vomiting, abdominal cramps, fever and/or bloody stool (Nsoesie *et al*., 2014). The illness occurs due to the consumption of contaminated or toxic food (Xue and Zhang, 2013). Foodborne disease is a global issue and has been reported worldwide. Incidence rates have been reported to be 1210 cases per 100,000 inhabitants in France, 2600 cases per 100,000 in the United Kingdom, and more than 25,000 cases per 100,000 inhabitants in Australia and the United States (Teisl and Roe, 2010). Malaysia however, reported a low incidence rate of 48 cases per 100,000 populations (Ministry of Health, Malaysia, 2014) but cases of foodborne illness in Malaysia usually go unreported because a chain of events need to be addressed first before it is brought to the authority (Soon *et al*., 2011) therefore, the actual rate is likely higher. In addition, the diarrhoeal disease has contributed 3% mortality globally (World Health Organization, 2014) and should be a cause for concern. Foodborne pathogens can be transmitted at different stages of food preparation. Typical pre-harvest sources of contamination are animal faeces, animals already infected by pathogenic microorganisms and meat that comes into contact with animal intestine, skin or fur (European Food Safety Authority, 2014).

A lot of studies have been done to determine the presence of pathogenic bacteria in food and food contact surfaces. Methods of FBB identification have also been improved. The traditional method typically uses a biochemical test for bacterial detection and eventually, more accurate techniques like polymerase chain reaction (PCR) are created for detection of FBB DNA (Naravaneni and Jamil, 2005). Instruments like pulsed-field gel electrophoresis (PFGE) are proven to be important in the field of food safety because they can monitor the spread of bacterial strains, provide pieces of evidence for epidemiological investigations and aid in the detection of the outbreak (Félix *et al*., 2014). DNA microarray allows for the detection and quantification of foodborne pathogens, and this method was used to identify the low concentration of FBB simultaneously from food sample (Kupradit *et al*., 2013). To this extent, methods adopted to identify foodborne pathogens are constantly being developed to generate more reliable and faster results.

The pyrosequencing analysis of 16S rRNA gene amplicon, is also a technique widely used to study microbial diversity from various samples such as, blood (Kaewkong *et al*., 2014), soil (Acosta-Martínez, *et al*., 2008) and food (Nam *et al*., 2012 & Masoud *et al*., 2012). Pyrosequencing has been used in the field of food science to identify microorganisms in samples like Japanese traditionally fermented sushi (Koyanagi *et al*., 2013), pearl-millet slurry (Humblot and Guyot, 2009), fish (Jung *et al*., 2014) and vegetable compartment of the refrigerator (Joen *et al*., 2013). However, no studies have been done to determine microbial

community of kitchen utensils like cutting board using this technique. The objective of this research was to analyze the microbial community that presents on 26 cutting boards obtained from different kitchens of various grades of restaurants in Seri Kembangan, Malaysia and to identify FBB present on the cutting boards.

4.2 Result

4.2.1 Total reads and operational taxonomy units (OTUs) of bacteria

The bacterial DNA obtained was enough and suitable for the pyrosequencing analysis. Total reads of bacteria isolated from cutting board from different grades of food premises are shown in Table 4.1. The pyrosequencing analysis managed to identify 452,401 reads of bacteria from 26 samples of cutting board. All samples contained a high diversity of microbial community. The numbers of reads obtained from this study ranging from 1 to more than 10,000 reads. Sample cb18 contained the highest number of OTUs (508 OTUs) and the lowest number was from sample cb21 (115 OTUs); both from grade B food premises. Sample cb11 from grade C food premise showed the highest number of reads (42,075 reads; 234 OTUs) whereas the least dominated (1,882 reads; 176 OTUs) was sample cb20 from grade B food premise. Nevertheless, the number of reads from different premise grades varied and was uninfluenced by the grade itself. From the data, it can be observed that sample cb26 which was from grade A food premise contained 20,076 reads (268 OTUs) while cb10 from grade C premise contained 7595 reads (200 OTUs) (Table 4.1).

Table 4.1 Total reads and operational taxonomy units (OTUs) of bacteria on cutting board samples

Samples name	Premise grades	Total reads	Total OTUs
cb1	A	9835	168
cb2	A	14881	239
cb3	B	18031	180
cb5	B	13023	179
cb6	B	8019	202
cb7	A	15036	225
cb8	C	13093	182
cb9	B	23052	354
cb10	C	7595	200
cb11	C	42075	234
cb12	B	28797	270
cb13	B	26754	117
cb14	B	26714	226
cb15	B	3568	178
cb16	B	26779	168
cb17	B	4024	216
cb18	B	29010	508
cb19	B	9364	142
cb20	B	1882	176
cb21	B	18436	115
cb22	B	11712	189
cb23	A	16783	192
cb24	B	11922	175
cb25	C	13958	192
cb26	A	20076	268
cb27	B	37982	362
Total		452401	

4.2.2 Relative abundance of the bacterial phyla and genera

Figure 4.1 shows the relative abundance of the bacterial phylum and the differences between all samples. *Proteobacteria, Firmicutes* and *Bacteroidetes* were identified as the most abundant phyla in the samples. Proteobacteria can be found to dominate every sample except for samples cb13 and cb21 that contained 63% and 92% of *Firmicutes* respectively. Samples cb8, cb11 and cb18 also contained a high percentage of Bacteroides which were 45%, 40% and 32% respectively.

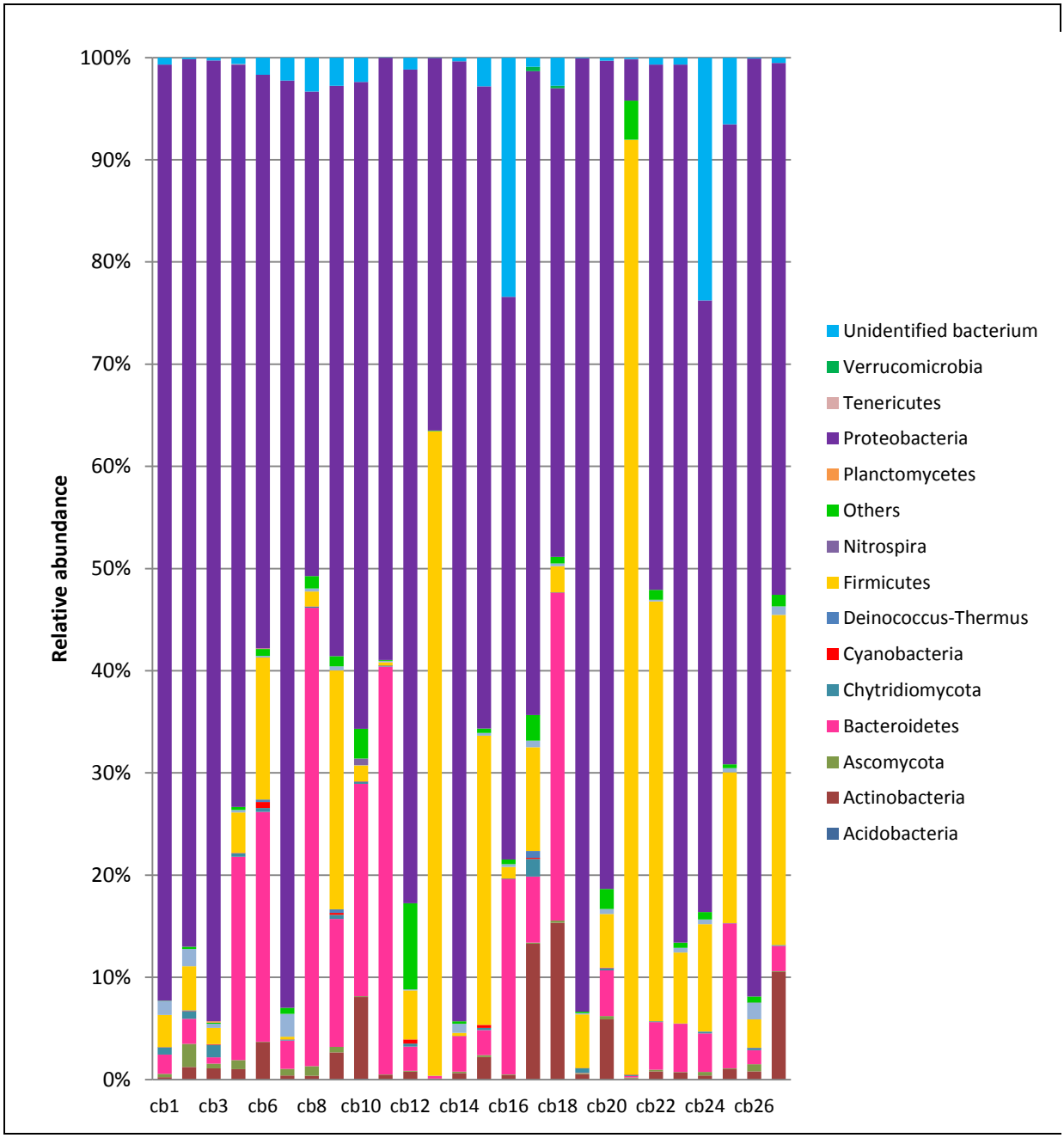


Figure 4.1 Taxonomy summary of the phylum (percentage) and comparison with relative abundances in other samples

The analysis also classified 363 genera and one unidentified genus. From this, 32 genera of the highest abundance were chosen and compared based on their percentages in every sample (Figure 4.2). The most prevalent genus from each sample varied, and it was observed that two genera dominated more than 80% of the total population, which were *Pseudomonas* from sample cb3 (grade B) and *Weisella* from sample cb21 (grade B) accounting for 93% and 81%, respectively. Some samples also had one genus accounting for more than half of its total population, like samples cb1, cb26 and cb23 which contained 65% *Acinetobacter*, 54% *Enterobacter* and 68% *Pseudomonas*, respectively. In

addition, sample cb13 (grade B) contained 63% of *Weisella*. Other samples were dominated by two genera of bacteria like sample cb2 (grade A) which had 42% of *Acinetobacter* and *Pseudomonas* respectively. Some samples contained high percentage of unidentified bacterium like cb16 and cb24 (grade B) which were 25% and 26% respectively. Additionally, this study revealed that *Enterobacter* and *Pseudomonas* were commonly found in most of the samples.

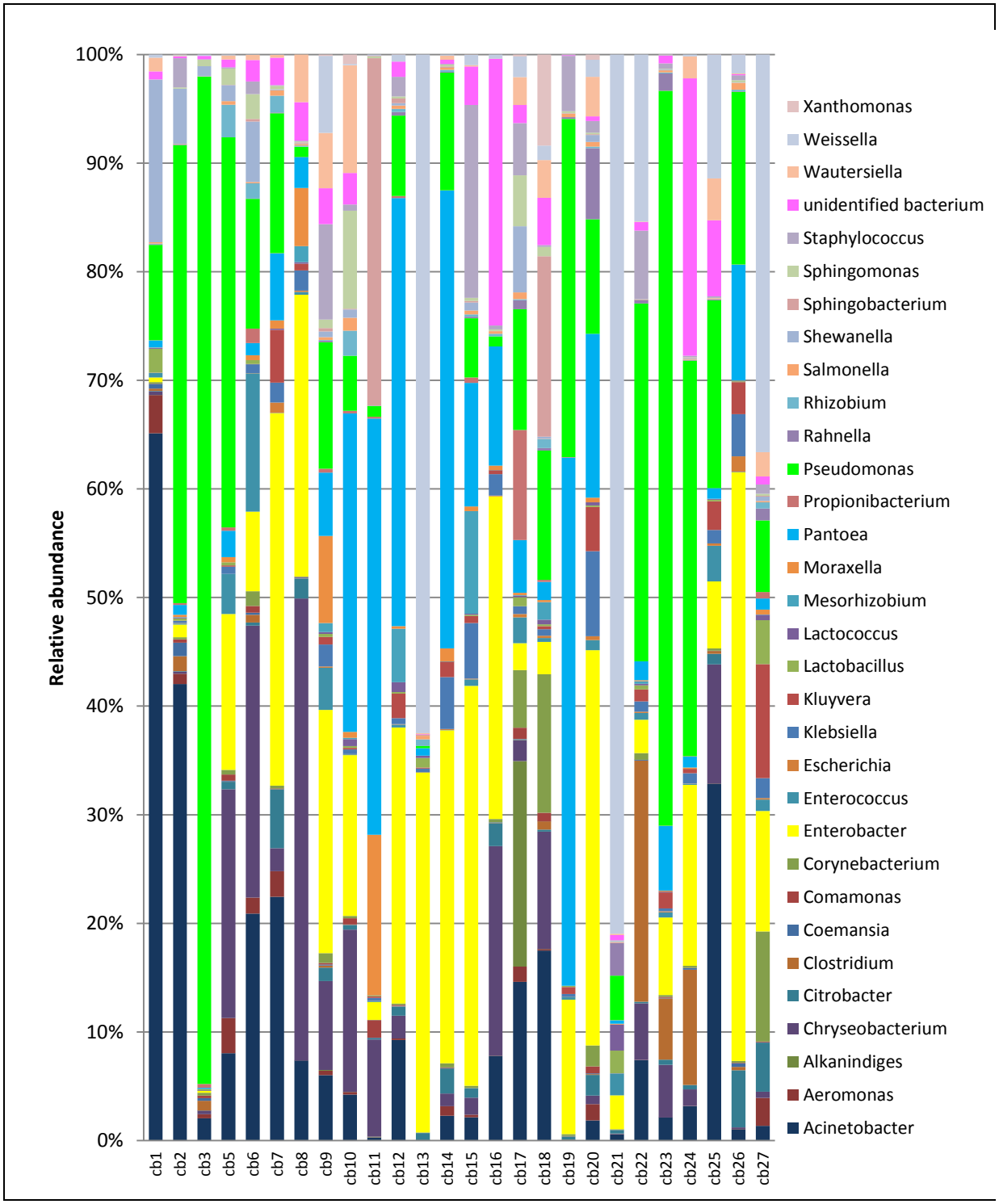


Figure 4.2 Taxonomy summary of 32 genera (percentage) and comparison with relative abundances in other samples

Among more than 1000 OTUs obtained from this analysis, 40 OTUs with high appearance frequencies were recorded (Table 4.2). OTUs closely related to *Enterobacter* sp., *E. aerogenes*, *Pseudomonas sp*. and *P. putida* were found in all samples (100% appearance frequencies). Most of the samples with more than 90% appearance frequencies were found to belong to the phyla of *Proteobacteria*.

Table 4.2 Bacteria with high appearance frequencies

No.	Bacteria	Appearance frequencies (%)
1	*Enterobacter aerogenes*	100
2	*Enterobacter* sp.	100
3	*Pseudomonas putida*	100
4	*Pseudomonas* sp.	100
5	*Acinetobacter johnsonnii*	96.2
6	*Citrobacter* sp.	96.2
7	*Klebsiella pneumoniae*	96.2
8	*Pantoea calida*	96.2
9	*Pantoea stewartii*	96.2
10	*Pseudomonas fulva*	96.2
11	*Pantoea ananatis*	92.3
12	*Acinetobacter calcoaceticus*	88.5
13	*Corynebacterium variabile*	88.5
14	*Klebsiella* sp.	88.5
15	*Leuconostoc pseudomesenteroides*	88.5
16	*Staphylococcus* sp.	88.5
17	*Clostridium* sp.	84.6
18	*Moraxella osloensis*	84.6
19	*Pseudomonas knackmussii*	84.6
20	*Weissella paramesenteroides*	84.6
21	*Enterobacter gergoviae*	80.8
22	*Pseudomonas fluorescens*	80.8
23	*Wautersiella falsenii*	80.8
24	*Acinetobacter septicus*	76.9
25	*Chryseobacterium hominis*	76.9
26	*Chryseobacterium* sp.	76.9
27	*Chryseobacterium taihuense*	76.9
28	*Hafnia alvei*	76.9
29	*Kluyvera ascorbata*	76.9
30	*Rahnella aquatilis*	76.9
31	*Wautersiella* sp.	76.9
32	*Weissella cibaria*	76.9
33	*Shewanella baltica*	73.1
34	*Kurthia gibsonii*	61.5
35	*Enterococcus faecalis*	53.8
36	*Mesorhizobium* sp.	50.0
37	*Sphingobacterium multivorum*	46.2
38	*Sphingomonas phyllosphaerae*	46.2
39	*Lactobacillus vaginalis*	42.3
40	*Xanthomonas sp.*	23.1

4.2.3 Determine bacterial number through quantitative PCR

Next, we estimate the total bacterial population by qPCR assay of 16SrDNA (Table 4.3). Sample cb25 from grade C food premise contained the highest bacterial number (1.3×10^8 bacterial cells/cm^2) whereas the lowest bacterial number was from sample cb6 from grade B food premise (2.5×10^4 bacterial cells/cm^2). This study also identified 13 related OTUs of FBB (Table 4.4). The table shows that *Salmonella enterica* had the highest appearance frequency (92.3%), followed by *Cronobacter sakazakii* (88.5%) and *Hafnia alvei* (76.9%).

Table 4.3 Number of bacteria on CB samples

Samples name	Number of bacteria per cm^2
cb1	2.4×10^7
cb2	2.2×10^6
cb3	1.5×10^5
cb5	1.2×10^5
cb6	2.5×10^4
cb7	5.4×10^6
cb8	5.0×10^5
cb9	6.8×10^5
cb10	1.3×10^6
cb11	7.6×10^7
cb12	7.6×10^6
cb13	1.6×10^7
cb14	5.3×10^5
cb15	1.2×10^5
cb16	2.6×10^6
cb17	6.2×10^6
cb18	5.7×10^6
cb19	1.9×10^6
cb20	6.0×10^6
cb21	3.9×10^7
cb22	5.6×10^6
cb23	3.1×10^6
cb24	9.6×10^5
cb25	1.3×10^8
cb26	7.3×10^6
cb27	2.2×10^7
Total	3.6×10^8

Sample cb27 from grade B premise had the highest number of OTUs closely related to FBB (1.8×10^6 bacterial cells/cm^2) followed by cb25 (2.3×10^5 CFU/cm^2) from grade C premise and cb26 (1.6×10^5 bacterial cells/cm2) from grade A premise. The lowest number was from samples cb3 and cb6 (7.3×10^1 and 4.6×10^1 bacterial cells/cm^2), both from grade B premises. The finding showed a body of interesting data where samples cb7 and cb26 from grade A

food premises contained 2.9 × 10^3 and 2.2 × 10^3 bacterial cells/cm2 of *E. coli* O157:H7 related OTU. This is because, grade A premises are usually associated with having great hygiene and clean environment but these samples contained more *E. coli* O157:H7 related OTU compared to the samples from lower grades of premises. These cutting boards also contained high number of *E. coli* related OTU (3.8 × 10^4 and 8.7 × 10^4 bacterial cells/cm^2) compared to other grades of premises. Sample cb12 from grade B premise contained a high number of *B. cereus* related OTU (6.1 × 10^3 bacterial cells/cm^2). Further multiplication of this organism will cause the production of heat resistant spores and cause serious illness. In this study, other types of FBB like *Campylobacter jejuni*, *Listeria monocytogenes*, *Shigella,* and *Vibrio cholera* were not identified.

The percentage of FBB related species over total reads on each cutting board was also determined (Table 4.4). All samples contained less than 2.2% of FBB related species in each sample except for samples cb15 and cb27 from grade B premises which contained 11.0% and 8.5% FBB related species, respectively. This low percentage might explain why some food premises manage to avoid foodborne illnesses even though they have poor sanitation level.

Table 4.4 Bacterial cells/cm^2 of foodborne bacteria

Foodborne bacteria	Samples and Premise Grades												
	cb1	cb2	cb3	cb5	cb6	cb7	cb8	cb9	cb10	cb11	cb12	cb13	cb14
	A	A	B	B	B	A	NG	B	C	C	B	B	B
Bacillus cereus	0	1.5×10^2	0	0	6.2	1.1×10^3	0	2.6×10^2	0	0	6.1×10^3	0	5.9×10^1
Cronobacter sakazakii	0	3.0×10^2	0	4.2×10^2	1.2×10^1	3.7×10^4	2.5×10^3	2.0×10^3	2.6×10^3	2.3×10^4	4.8×10^3	3.0×10^4	2.1×10^3
Cronobacter turicensis	0	0	0	0	0	3.6×10^2	3.8×10^1	0	0	0	0	0	7.9×10^1
Escherichia coli	4.8×10^3	6.0×10^2	0	0	0	3.8×10^4	3.4×10^2	3.5×10^2	0	3.6×10^3	0	1.2×10^3	5.9×10^1
Escherichia coli O157:H7	0	0	0	0	0	2.9×10^3	3.8×10^1	0	0	1.8×10^3	0	5.8×10^2	0
Hafnia alvei	2.4×10^4	0	6.5×10^1	0	3.1	4.3×10^3	3.8×10^1	2.9×10^1	3.5×10^2	7.0×10^4	8.0×10^2	0	3.2×10^2
Kurthia gibsonii	0	0	0	0	0	3.6×10^2	4.3×10^3	4.5×10^3	1.1×10^3	5.4×10^3	7.3×10^4	0	0
Salmonella bongori	0	0	0	0	0	0	0	0	0	0	0	5.8×10^2	5.9×10^1
Salmonella enterica	0	1.0×10^3	0	3.5×10^2	2.5×10^1	2.1×10^4	6.9×10^2	9.4×10^2	5.6×10^3	2.5×10^4	1.1×10^4	5.1×10^4	7.7×10^2
Salmonella paratyphi	0	0	0	0	0	0	3.8×10^1	8.8×10^1	0	0	0	0	2.0×10^1
Salmonella sp.	7.2×10^3	0	0	1.9×10^1	0	2.2×10^3	3.8×10^1	2.1×10^2	7.4×10^3	0	5.6×10^3	5.8×10^2	2.0×10^1
Salmonella typhi	1.7×10^4	0	8.1	0	0	7.2×10^2	0	1.8×10^2	0	0	4.5×10^3	0	2.0×10^1
Salmonella typhimurium	0	0	0	0	0	0	0	2.9×10^1	1.8×10^2	0	0	0	0
Yersinia enterocolitica	2.4×10^3	0	0	0	0	1.1×10^3	0	5.9×10^1	0	7.2×10^3	0	0	3.9×10^1
Total	5.5×10^4	2.1×10^3	7.3×10^1	7.9×10^2	4.6×10^1	1.1×10^5	8.0×10^3	8.7×10^3	1.7×10^4	1.4×10^5	1.1×10^5	8.3×10^4	3.5×10^3
Percentage of foodborne bacteria to total number of bacteria on each cutting board	0.23	0.09	0.05	0.65	0.19	2.01	1.60	1.29	1.29	0.18	1.38	0.53	0.67

Table 4.4 *(Continued)*

Foodborne bacteria	Samples and Premise Grades													Appearance frequencies (%)
	cb15	cb16	cb17	cb18	cb19	cb20	cb21	cb22	cb23	cb24	cb25	cb26	cb27	
	B	B	B	B	B	B	B	B	A	B	C	A	B	
Bacillus cereus	1.3×10^{2}	4.9×10^{2}	0	0	0	0	2.1×10^{3}	0	1.8×10^{2}	8.0×10^{1}	0	2.2×10^{3}	5.7×10^{2}	50.0
Cronobacter sakazakii	1.7×10^{2}	1.7×10^{4}	0	9.8×10^{2}	1.0×10^{3}	1.3×10^{4}	2.1×10^{3}	2.8×10^{3}	6.8×10^{3}	7.2×10^{2}	9.8×10^{4}	2.3×10^{4}	1.4×10^{4}	88.5
Cronobacter turicensis	0	2.0×10^{2}	0	0	0	0	0	0	0	0	0	0	1.1×10^{3}	19.2
Escherichia coli	0	1.5×10^{3}	7.7×10^{3}	5.7×10^{3}	0	3.2×10^{2}	0	4.7×10^{2}	0	2.4×10^{2}	0	8.7×10^{4}	2.2×10^{4}	61.5
Escherichia coli O157:H7	0	0	0	0	0	0	0	0	0	0	0	2.2×10^{3}	0	19.2
Hafnia alvei	0	5.9×10^{2}	0	1.4×10^{3}	8.0×10^{2}	2.6×10^{4}	0	4.7×10^{3}	3.7×10^{2}	8.0×10^{1}	4.5×10^{4}	1.8×10^{3}	1.7×10^{6}	76.9
Kurthia gibsonii	1.3×10^{4}	0	1.1×10^{4}	1.6×10^{4}	0	3.2×10^{3}	4.3×10^{3}	7.1×10^{4}	1.8×10^{2}	8.0×10^{1}	0	7.3×10^{2}	3.5×10^{4}	61.5
Salmonella bongori	0	0	0	0	0	0	0	0	1.8×10^{2}	0	0	1.5×10^{3}	0	15.4
Salmonella enterica	2.7×10^{2}	4.6×10^{3}	1.5×10^{4}	7.8×10^{2}	5.4×10^{3}	9.6×10^{3}	6.4×10^{3}	1.9×10^{3}	4.6×10^{3}	4.8×10^{2}	8.9×10^{3}	2.9×10^{4}	9.1×10^{3}	92.3
Salmonella paratyphi	0	9.8×10^{1}	0	0	2.0×10^{2}	0	0	0	0	0	0	1.5×10^{3}	5.7×10^{2}	26.9
Salmonella sp.	0	1.2×10^{3}	1.5×10^{3}	0	0	3.2×10^{3}	2.1×10^{3}	0	7.4×10^{2}	8.0×10^{1}	8.9×10^{3}	2.5×10^{3}	1.6×10^{3}	69.2
Salmonella typhi	6.7×10^{1}	0	1.5×10^{3}	0	0	6.4×10^{3}	0	0	0	8.0×10^{1}	3.6×10^{4}	6.5×10^{3}	1.1×10^{3}	50.0
Salmonella typhimurium	0	3.9×10^{2}	1.5×10^{3}	0	0	0	0	0	0	0	0	0	0	15.4
Yersinia enterocolitica	0	9.8×10^{1}	0	0	0	0	2.1×10^{3}	9.5×10^{2}	1.1×10^{3}	0	0	1.1×10^{3}	8.5×10^{3}	42.3
Total	1.3×10^{4}	2.7×10^{4}	3.8×10^{4}	2.5×10^{4}	7.4×10^{3}	6.4×10^{4}	1.9×10^{4}	8.2×10^{4}	1.4×10^{4}	1.8×10^{3}	2.3×10^{5}	1.6×10^{5}	1.8×10^{6}	
Percentage of foodborne bacteria to total number of bacteria on each cutting board	11.04	1.01	0.62	0.44	0.40	1.06	0.05	1.48	0.46	0.19	0.19	2.18	8.52	

4.2.4 Alpha diversity

Figure 4.3 shows the rarefaction curves of the samples and Table 4.5 shows the mean value of species richness based on observed species at 1506 reads. The statistical analysis shows that there was no significant different of microbial community between premises grade A, B, and C.

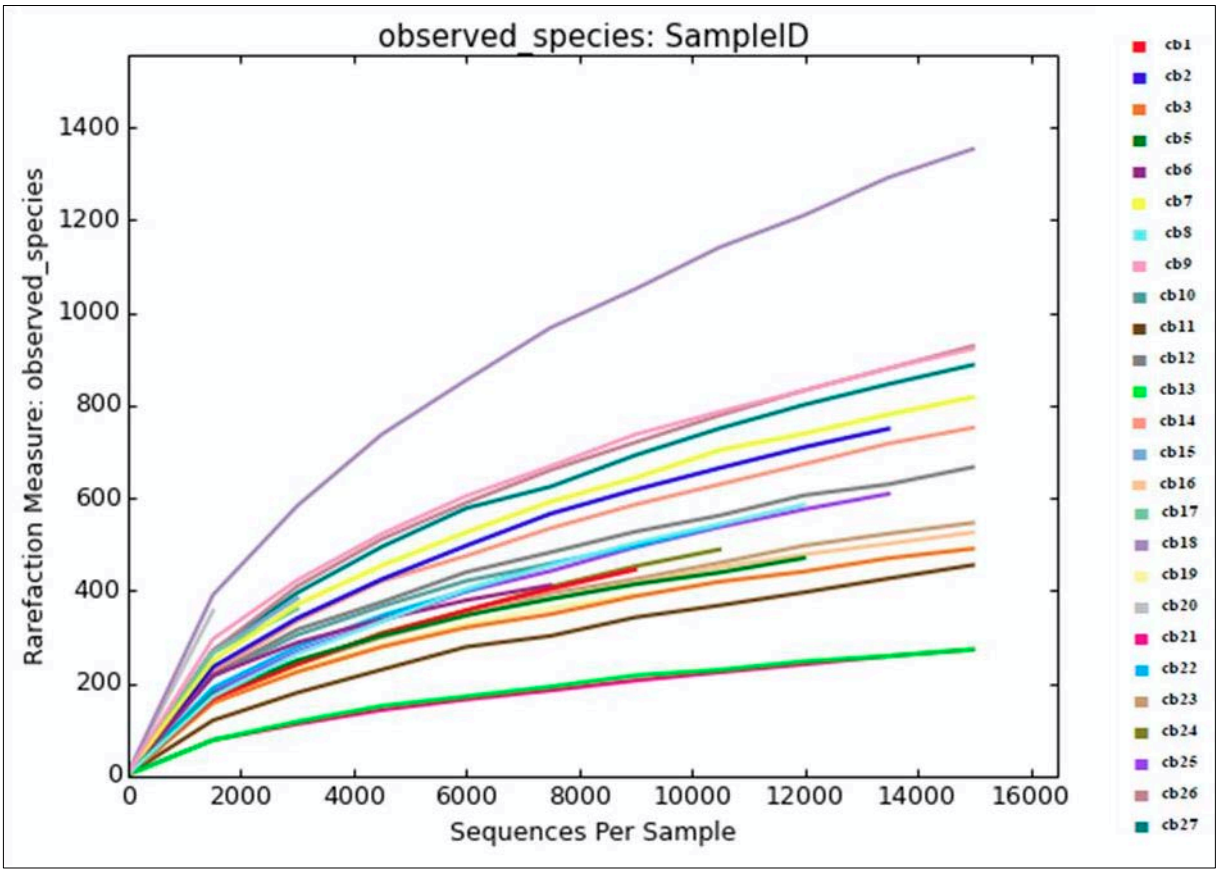


Figure 4.3 Alpha diversity rarefaction curves of observed species

Table 4.5 Mean (SD) observed species at n = 1506 based on premise grades

Variable	Mean (SD) observed species			*t* statistic (df)	*p* value
	A	B	C		
Premise grades	217.4 (49.0)	216.1 (85.7)	172.4 (39.1)	0.572 (2, 23)	0.572

*One-way ANOVA

4.2.5 Beta diversity

Furthermore, principal coordinates plot (Figure 4.4) of the bacterial community composition based on the three point cleanliness scale was performed. Using weight Unifrac bacterial community in all grade A food premises (cb1, cb2, cb7, cb23 and cb26) clustered together with bacterial communities in grade B (cb3, cb14, cb16, cb18, cb19 and cb24) and C (cb10) food premises suggesting that they had similar species abundance. However, bacterial communities of other samples from grade B and C food premises differ substantially from one another, implying that they were quantitatively different and more diverse.

A different result was shown by the beta diversity of FBB in all samples. Foodborne bacteria from A, B and C premises did not cluster within the same group but rather, scattered across the plot and demonstrated similarity with different FBB from different premise grades. This result suggesting that, different premise grades did not show any specific trend on the presence of FBB on the CB.

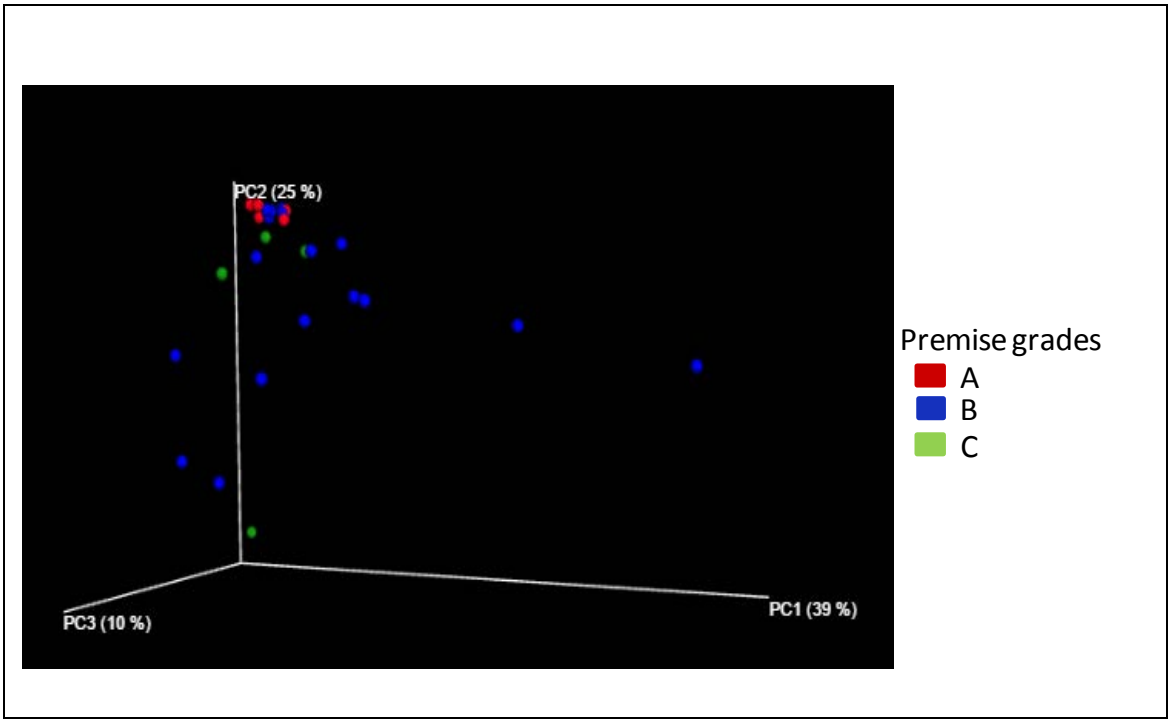


Figure 4.4 Principal coordinates plots comparing the relative abundance of different bacterial taxa in samples from different premise grades, tested using and weight UniFrac

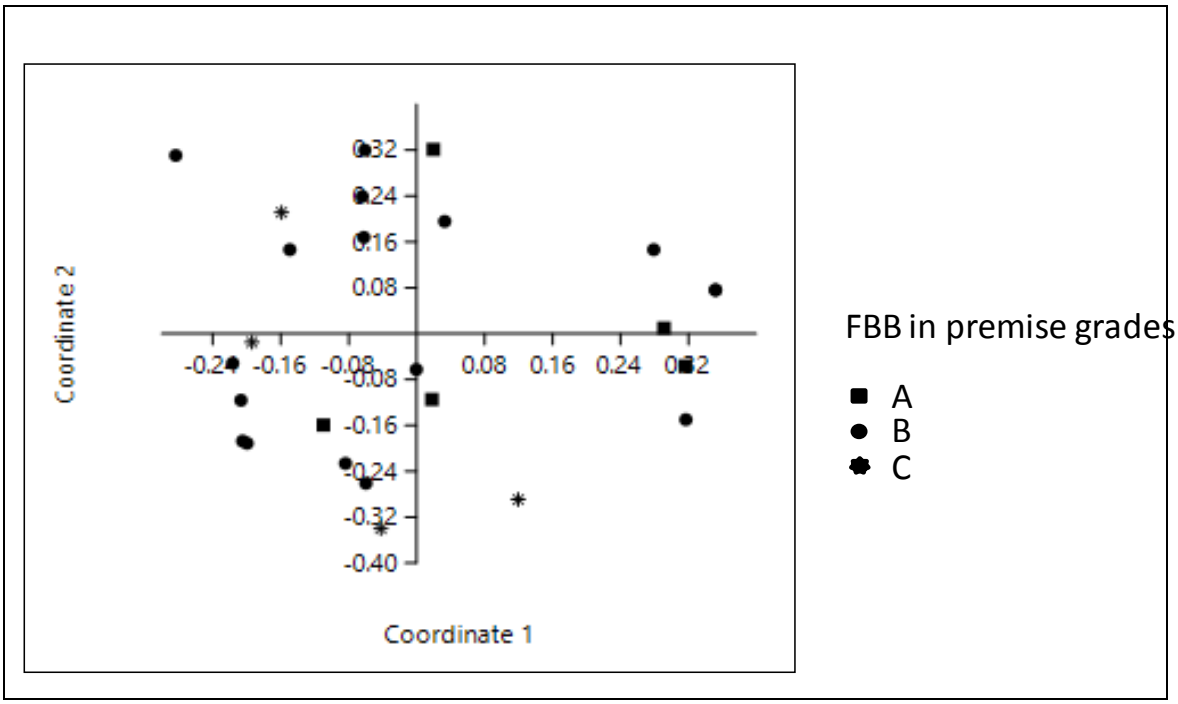


Figure 4.5 Principal coordinates plots comparing the relative abundances of FBB in samples from A, B and C food premises

4.2.6 Correlation analysis

In addition, the total number of bacteria and the number of FBB are not significantly correlated with the grades of food premises (Table 4.6). This means that the high grade food premises contain approximately the same microbial community and the same number of bacteria as low grade food premises.

Table 4.6 Correlation between premise grades and total number of bacteria and number of foodborne bacteria

	Premise grades	
	r	*p* value*
Total number of bacteria	-0.018	0.931
Number of foodborne bacteria	0.021	0.919

* Spearman correlation

4.3 Discussion

4.3.1 Molecular analysis to study food safety

Molecular analysis has been a reliable method to study FBB in food and food related samples. A study by Bang *et al*. (2013), used microarray to detect low population of *Listeria monocytogenes* (3-4 log CFU/mL) with the presence of high number of other bacteria in milk. Microarray can also identify genes that are responsible for illnesses. Using this technique, Chen *et al*. (2005) discovered virulence and antimicrobial resistance genes in *Salmonella* and *E. coli* isolated from retail ground meat and food animals. Osés *et al*. (2013) used denaturing gradient gel electrophoresis (DGGE) and real-time PCR to determine the number of pathogenic bacteria in different atmospheric conditions. The study discovered that high carbon dioxide atmosphere is suitable for the storage of fresh cut suckling lamb due to its inhibitory effect of most bacteria. Rodriguez-Lazaro *et al*. (2014) also discovered the effectiveness of real-time PCR to detect 2-4 *Salmonella* spp. CFU per 25 g sample of meat, dairy and vegetable food products in less than 21 h. In another study, Martín *et al*. (2014) used multilocus sequence typing (MLST) to determine the diversity of *L. monocytogenes* and contamination routes across different meat processing plants.

Pyrosequencing analysis of 16S rRNA gene amplicon has been recently used to study microbial community in various samples and has been reported to identify FBB rapidly. A study by Joen *et al*. (2013) discovered that the microbial population in the refrigerator is highly colonized by four phyla, like *Proteobacteria, Firmicutes, Actinobacteria* and *Bacteroides*. They also discovered that *Propionibacterium acnes*, *Bacteroides vulgates* and *Staphylococcus epidermidis* which can be found on human skin also present in the refrigerator, suggesting the probability of contamination. Using pyrosequencing technique, Koyanagi *et al.* (2013) discovered that the population of *Lactobacillus* drastically increased during the fermentation of medieval Sushi while *Staphylococcus* and *Bacillus* population decreased.

In another study, Jung *et al*. (2014) found out that the highest genus of bacteria in Korean fermented fish is *Lactobacillus*, with high production of metabolites like fructose, glucose and maltose. A few studies were able to detect a very low number of *Campylobacter* (10^2 CFU/mL), *Bacillus antrachis* (6 CFU/g and 6 CFU/mL) and *Salmonella* (100 isolates) with less than 7 hours to complete the assays (Amoako *et al*., 2013; Li *et al*., 2012 and Oakley *et al*., 2012). 454 platform is a next generation sequencing that has been widely used in the field of genomic and molecular biology. This platform delivers sequencing reads up to 1000 base pairs in length. On the other hand, another instrument called Miseq, provides similar result with less read length. Miseq, a high troughput sequencer would provide much higher resolution due to the depth of sequencing, for almost one tenth of the cost of 454 platform.

4.3.2 Microbial diversity

The effect of different environments or nutrients towards species richness has been widely studied (Liu *et al*., 2013; Gomes *et al*., 2013 and Buosi *et al*., 2011). However, the analysis of samples from kitchen environments has been very limited. Here, we firstly reported on the microbial community isolated from kitchen cutting board collected from different grades of food premises. The α- and β- diversity (Table 4.5, Figure 4.4 and Figure 4.5) of this study showed that microbial abundances on cutting boards from different grades of food premises were high and FBB number were not correlated with the hygiene level as the physical appearance of food premises. The PcoA plot shows that bacterial community from all grade A premise, cluster together with some of B and C premises. The study has identified that material of CB and food items were not the same, thus these could not be the factor for the same community composition. However, based on the observation during CB collection, we identified that the food handlers might be contributing to the same composition. This is because, in lower premise grades, food handlers came from different background and country of origin, whereas in high premise grades, most of the food handlers were local people, suggesting the contribution of the same community composition. Nonetheless, research has shown that food handlers can easily spread microorganism in the kitchen (Linscott 2011).
Specific β- diversity for FBB performed in this study, showed no definite trends in the presence or absence of the FBB based on the premise hygiene level. The statistical test also displayed no significant different of bacterial number between A, B and C premises. This signifies that all premises might be having the same FBB and that the risk levels are the same between these premises. Thus, these data strengthen the fact that all food premises, regardless of their status, have the potential to cross-contaminate food items via cutting boards. In addition, microbial community that presents on the cutting boards can be mostly found in the environment and act as human microflora which eases their transmission.

Furthermore, α- and β- diversity have been widely used in other research fields especially to study species richness. Xu *et al*. (2014), discovered that even though the number of microfauna are higher at a depth of 1 m compared to 3 m, there was no significant different between the value of the species richness. Feldman *et al*. (2012), found out that viruses that infect fungi are less specialized than was previously thought. Using beta diversity, Dunn *et al*. (2013) discovered that presence of dogs had a significant effect on bacterial community composition in several areas within homes. They also found a significant relationship between the types of bacteria found outside the home and inside the home.

To add, several bacteria were found to dominate the samples, like *Pseudomonas* which can be commonly found in soil (Chien *et al*., 2013), and *Enterobacter aerogenes* which is a member of normal flora in the gastrointestinal tract (Chen *et al*., 2014 & van Rossem *et al*., 2007). Opportunistic microorganisms like *Pseudomonas aeruginosa, P. fulva* and *P. putida,* can cause hospital-acquired infections like cystic fibrosis and bacteremia (Valderrey *et al*., 2010, Liu *et al*., 2014) and *Enterobacter aerogenes* can cause

blood infection (Song *et al*., 2010). Other bacteria that can harm to human health have also been identified, for instance *Klebsiella pneumoniae* which can cause pneumonia, *Lactobacillus vaginalis*, a member of lactic acid bacteria which can cause vaginitis. This bacterium is originated from the vagina and has a protective effect against bad bacteria (Embley *et al*., 1989). The existence of *L. vaginalis* on the cutting board shows poor hygiene and sanitation of food handlers which transfer this bacterium from their genital area to kitchen utensils via unclean hands.

4.3.3 Foodborne bacteria in the samples

In this study *Bacillus cereus* was detected from half of the total samples. This organism is usually associated with rice contamination (Kim *et al*., 2014) with an infectious dose of 10^4-10^5 CFU/mL (Pina-Pérez *et al*., 2012). *B. cereus* spores are able to survive under environmental stresses like air-drying, heat, high pressure, UV light and acidity (Abbas *et al*., 2014). These spores will germinate when the conditions become suitable, and cause foodborne illnesses with symptoms like diarrhoea, vomiting and abdominal pain (Carlin *et al*., 2010). Additionally, only a few spores are required to cause food spoilage (van Melis *et al*., 2014) thus, food temperature should be maintained high or low to avoid the growth of this bacterium.

The study also points to the existence *of E. coli* O157:H7 related OTU on cutting boards collected from clean food premises. Cattles and other ruminants, which are widely used as cooking ingredients in the kitchen are usually the reservoirs for this serotype of *E. coli* (Ayaz *et al*., 2014). The population of this bacterium isolated from grade A food premises is higher compared to other premises. The infectious dose for this pathogen is about 10-100 organisms per contaminated food (Charimba *et al*., 2010 and Marouani-Gadri *et al*., 2010), thus, following the improper handling and the occurrence of temperature abuse, this strain can proliferate and contaminate food products and pose high risk to human consumption.

In this study, the FBB are most frequently found belong to the genus of *Salmonella*. These bacteria have been known to cause salmonellosis with lower infective dose (Teunis *et al*., 2010). In Malaysia food handlers are compelled to take typhoid injection to avoid becoming a carrier and contaminate food with *Salmonella*. These results reveal an alarming data of the number of *Salmonella enterica* related OTU on the samples from grade A food premises ranging from 4.6×10^3 to 2.9×10^4 bacterial cells/cm^2).

Some bacteria that are not well recognized as foodborne pathogens such as *Cronobacter sakazakii*, *Cronobacter turicensis*, *Hafnia alvei* and *Kurthia gibsonii* related OTUs have been found in this study. *C. sakazakii* and *C. turicensis* can be isolated from milk, cheese, dried foods, meats, vegetables, rice, bread, tea, herbs, spices, powdered infant formula and water. They can cause infections like neonatal meningitis, septicemia, and enterocolitis in neonates (Emami *et al*., 2011). *H. alvei* is commonly found in soil, water, and food, where these bacteria constitute normal gastrointestinal flora of mammals and birds (Bobko *et al*.,

2013). This bacterium can cause acute gastroenteritis and extra-intestinal disease (Katzenellenbogen *et al*., 2013). *Kurthia gibsonii* is another bacterium that is rarely known as foodborne pathogen. This bacterium has been isolated from prawn as well as meats and meat products, and the ingestion of this microorganism can cause diarrhoea (Paul *et al*., 2012 and Stackebrandt *et al*., 2006).

Staphylococcus aureus, Clostridium perfringens and *C. botulinum* are other species of bacteria that cause foodborne illnesses. *S. aureus* produces heat-stable enterotoxins (Wu and Su, 2014), which means that, heating might kill the microorganism but a person might still experience illness due to this toxin. C*. perfringens* also produces toxins and is classified into five groups of A, B, C, D or E based on the production of alpha (α), beta (β), epsilon (ε), and iota (ι) toxins (Milach *et al*., 2012). The unsafe number of 10^5 CFU/g of this bacterium causes the production of enterotoxin (Eriksen *et al*., 2010) which leads to abdominal pain, nausea, vomiting and diarrhoea (Brynestad and Granum, 2002). Foodborne botulism caused by *C. botulinum* occurs due to the ingestion of food containing preformed botulinum toxin which is also capable of surviving cooking temperatures lower than 100 ºC (Neghina *et al*., 2010). Symptoms of illness include muscle weakness, dilated pupils and non-reaction to light, weakening jaw muscles and constipation (Tseng *et al*., 2009).

From this study, a very high diversity of microbial community existing on kitchen cutting boards has been identified. Further study can be done to determine the interaction between these microorganisms, their growth requirement and how they suppress or support the growth of other bacteria.

4.4 Conclusion

Pyrosequencing reveals a high diversity of microorganisms that present on cutting boards collected from different grades of food premises. This study has also discovered a few foodborne pathogens that have the potential to cause foodborne illnesses if cross contamination occurs from cutting board to food items. In addition, the grades of food premises have no apparent correlation with bacterial abundance and the number and species of microorganisms isolated from the cutting board. To add, the community composition of FBB based on premise grades also showed no similarity. Hence, to avoid foodborne illnesses, it is important that correct food handling is practised in every kitchen. Food handlers must wash hands and food contact surfaces when necessary, avoid cross contamination, cooking to proper temperature as well as keeping hot food and cold food the way they should be. Attention should be given to the fact that all grades of food premises have the same chance of introducing FBB from cutting boards to food items.

CHAPTER 5: ECOLOGICAL RELATIONSHIP OF MICROBIAL COMMUNITY AND FACTORS ASSOCIATED WITH THE PRESENCE OF FOODBORNE BACTERIA

5.1 Introduction

Foodborne pathogenic bacteria (FBB) are bacteria that are carried by food and can cause infections especially in the gastrointestinal system (Vongkamjan and Wiedmann, 2015; Wang *et al*., 2015). Most of the FBB belong to the *Enterobacteriaceae* family under the *Proteobacteria* phylum and are usually associated with bacteria like *Listeria monocytogenes, Escherichia coli, Salmonella, Campylobacter, Yersinia enterocolitica, Cronobacter* spp. and *Shigella* (Larsen *et al*., 2014; Baylis *et al*. 2011). Foodborne diseases have always been a concern during food consumption in food service establishments like restaurants, canteen, cafeteria and food stalls. Cleanliness of food premises is very important, however, repeated exposure of sanitizers to kitchen items will result in increasing the resistance of pathogens (Riazi and Matthews, 2011). In Malaysia, dirty condition or low grades of food premises, does not necessarily harm the consumers. This is because apart from human immune system, microflora in the gut aid in preventing the growth of pathogens which give benefits to the host (Kaiko *et al*., 2014; Crost *et al*., 2010), and microorganisms can also interact with the environment like soil and ocean (Venter *et al*., 2004; Rondon *et al*., 2000). Based on these earlier studies, microbial diversity was reported to be very high in human and in the environment; hence, this is the base for our research on microbial community analysis from CB samples.

Previous studies have also documented the effect of certain bacteria in avoiding the growth of pathogenic bacteria which can benefit humans, animals and plants. Orders *Clostridiales* and *Lactobacillales* are microbiota of healthy individual (Thiennimitr *et al*., 2012). *Lactobacillales* or lactic acid bacteria (LAB) have health-promoting activity (Ashraf *et al*., 2014) and the members of these orders contain a lot of antimicrobial compounds (O'Bryan *et al*., 2015). In addition, *Lactobacillales* was proven to support the growth of *Clostridiales* particularly genus *Caprococcus* which benefits the host (Ferrario *et al*., 2014). Apart from that, other beneficial bacteria are *Flavobacterium* sp. from order *Flavobacteriales,* which was found to produce flavocin, an agent of fungisitic and bacteriostatic (IuD *et al*., 1996). Additionally, *Pseudomonadales,* specifically genus *Pseudomonas* have biocontrol effect against alga and fungi and are able to produce its own metabolite like pseudophomins (Trotel Aziz, 2008; Pedras *et al*., 2003). In spite of this benefit, some bacteria can support the growth of bad bacteria through interspecies communication. For example, intestinal bacteria like *Salmonella* and *E. coli* are able to co-operate in order to enhance virulence gene expression and host colonization (Walters and Sperandio, 2006). *Yersinia enterocolitica* and *Serratia proteamaculans* can support the growth of *Salmonella* (Dourou *et al*., 2011) which could cause foodborne illness. Another study recorded a higher number of *Salmonella* in the presence of plant pathogen *Erwinia tracheiphila,* suggesting a synergistic interaction between these bacteria (Gautam *et al*., 2014). To top it off, *Citrobacter, Enterobacter, Escherichia, Klebsiella* and *Serratia,* have an almost similar antigenic

composition (Guentzel, 1996) which might allow interspecies communication and support the growth of each other.

This report emphasized on extended and deepened analysis from our previous study. Our preliminary research has shown that the level of cleanliness in food premises was not significantly associated with the number of foodborne bacteria on the cutting boards (Abdul-Mutalib *et al*, 2015). The earlier study also discovered the similarity of bacterial communities in all premises. In our previous study, we found that some samples contained a high proportion of FBB in a low total number of bacteria and a low proportion of FBB in a high total number of bacteria; although the numbers were lower than that of the infectious dose level. We also discovered that the bacteria which dominated the samples were different from one another. In this subsequent study, effects of the total number of bacteria on the occupation of FBB were discussed in depth.

Pyrosequencing analysis has been widely used to identify bacteria from food and food-related samples (Jung *et al*., 2014; Joen *et al*., 2013; Koyanagi *et al*., 2013; Nam *et al*., 2012). However, based on our knowledge, this is the first report that emphasizes on microbial community analysis in order to study potential health risk for consumers in Malaysia. We hope that the data obtained can give an overview of the safety level of local food service establishments, improve safety, and aid in the development of appropriate countermeasures. This paper aims to analyze the data statistically in order to identify which bacteria can effectively keep FBB number at a safe level and whether the high and a low number of background bacteria has any relationship with FBB. Furthermore, we would like to determine the other variables like types, usages and conditions of CB that might have any relationship with the growth of FBB.

5.2 Additional methodology

This study separated the samples into two groups of high and low bacteria number. The purpose of this procedure was to find out whether the total number of bacteria based on phylum, class, order and genus influences the percentage of FBB. The boundary was determined by trial and error method by entering values of 1.0×10^6 to 1.0×10^7. The data were then entered into a statistical package for calculation.

5.3 Result

5.3.1 Cutting board classification

A total of 26 CB were collected from various premises. Most of the premises were classified as moderately clean or grade B (Table 5.1). The number of plastic and wood CB were almost equal and were mostly (46.2 %) used to cut vegetables. Moreover, a majority (61.5 %) of them were classified as clean (no traces of food observed macroscopically).

Table 5.1 Classification of the cutting board samples

Variables	N (%)
Premise grades	
A	5 (19.2)
B	17 (65.4)
C	4 (15.3)
Types of CB	
Plastic	12 (46.2)
Woods	14 (53.8)
Usages of CB	
Common	7 (26.9)
Vegetables	12 (46.2)
Fruits	2 (7.7)
Chicken/beef	5 (19.2)
Physical conditions	
Dirty	6 (23.1)
Clean	16 (61.5)
Average	4 (15.4)

5.3.2 Grouping bacteria based on total number

Total background biota of less than 5.0×10^6 bacterial cells/cm^2 was selected for low bacteria group and total background biota of 5.0×10^6 bacterial cells/cm^2 and more was chosen for high bacteria group. Based on the total bacterial number of our 26 samples, the statistical package was able to perform analysis after 13 samples from each category were inserted for calculation, thus, 5.0×10^6 bacterial cell/cm^2 was chosen as the separation value. Lower or higher value than this caused too many samples in one category and too few samples in another, causing imbalance, thus failed to be analyzed. This value was also chosen because it allowed for high R^2 value in the correlation analysis.

5.3.3 Comparing the number of total bacteria and FBB

Statistical analysis showed that premise grade C contained significantly higher total bacterial number compared to grades A and B food premises. However, based on types, usages and physical conditions of CB, the number of bacteria was not significantly different (Table 5.2). From 26 samples, 13 were classified into high and low group respectively.

The mean difference of FBB in high and low bacteria group was also compared (Table 5.2). Overall, there was no significant difference of FBB in these two groups; however, mean difference based on other variables showed a different result. In high bacteria group, some samples contained significantly higher FBB compared to low bacteria group such as samples obtained from grades A and C food premises; plastic CB, common and chicken/beef CB, as well as dirty and average condition of CB.

Table 5.2 Mean difference of bacterial number, including FBB in high and low bacteria group based on premise grades, types, usages and conditions of CB

Variables	Mean (SD) bacterial number per cm^2		
	Total bacteria number	**FBB in high bacteria group**	**FBB in low bacteria group**
Overall		2.2×10^5 $(4.7 \times 10^5)^{Aa}$	7.9×10^3 $(8.0 \times 10^3)^{Aa}$
Premise grades			
A	8.3×10^6 $(8.7 \times 10^6)^{a}$	1.1×10^5 $(5.3 \times 10^4)^{Aa}$	8.1×10^3 $(8.4 \times 10^3)^{Ba}$
B	6.7×10^6 $(1.0 \times 10^7)^{a}$	2.8×10^5 $(6.2 \times 10^5)^{Aa}$	6.9×10^3 $(8.8 \times 10^3)^{Aa}$
C	5.1×10^7 $(6.0 \times 10^7)^{b}$	1.9×10^5 $(6.4 \times 10^4)^{Aa}$	1.3×10^4 $(6.4 \times 10^3)^{Ba}$
Types of CB			
Wood	1.7×10^7 $(3.3 \times 10^7)^{a}$	2.8×10^5 $(5.7 \times 10^5)^{Aa}$	4.6×10^3 $(5.5 \times 10^3)^{Aa}$
Plastic	1.0×10^7 $(2.2 \times 10^7)^{a}$	1.0×10^5 $(3.5 \times 10^4)^{Aa}$	1.0×10^4 $(9.0 \times 10^4)^{Ba}$
Usages of CB			
Common	1.0×10^7 $(1.5 \times 10^7)^{a}$	6.1×10^4 $(4.6 \times 10^4)^{Aa}$	7.5×10^2 $(9.6 \times 10^2)^{Ba}$
Chicken/beef	6.5×10^6 $(8.6 \times 10^6)^{a}$	9.4×10^5 $(1.2 \times 10^6)^{Aa}$	1.2×10^4 $(1.3 \times 10^4)^{Ba}$
Vegetables	2.1×10^7 $(3.9 \times 10^7)^{a}$	1.1×10^5 $(6.9 \times 10^5)^{Aa}$	9.8×10^3 $(4.8 \times 10^3)^{Aa}$
Fruits	9.2×10^5 $(5.9 \times 10^5)^{a}$	N/A	1.3×10^4 (6.4×10^3)
Conditions of CB			
Dirty	1.8×10^7 $(3.0 \times 10^7)^{a}$	1.0×10^5 $(4.3 \times 10^4)^{Aa}$	4.6×10^3 $(7.3 \times 10^3)^{Ba}$
Clean	1.2×10^7 $(3.0 \times 10^7)^{a}$	9.9×10^4 $(4.3 \times 10^4)^{Aa}$	9.8×10^4 $(9.2 \times 10^3)^{Aa}$
Average	1.6×10^7 $(1.8 \times 10^7)^{a}$	9.1×10^5 $(1.3 \times 10^6)^{Aa}$	5.5×10^3 $(2.8 \times 10^3)^{Ba}$

[a-a] Means (SE) with the same superscript in the same row (within the same group) is not significantly different (0.05 level)
[A-A] Means (SE) with similar superscript in the same column are not significantly different (0.05 level)

5.3.4 Percentage of FBB in high and low bacteria group

Bacterial population from each sample differs from one another (Table 5.3), with an average number of FBB in high bacteria group higher than that of low bacteria group. Although that was the case, the prevalence of FBB in low bacteria group was higher. The table also shows that most of the CB in the high bacteria group were made of wood, and used to handle vegetables.

Table 5.3 Bacterial cell number and the percentage of FBB

Low bacteria group		
Samples name*	Number of total bacteria per cm^2	Number of FBB per cm^2 (%)
2APG1	2.2×10^6	2.1×10^3 (0.09)
3BPG3	1.5×10^5	8.1 (0.01)
5BWG3	1.2×10^5	7.9×10^2 (0.65)
6BWG1	2.5×10^4	4.3×10^1 (0.17)
8CPF1	5.0×10^5	3.7×10^3 (0.74)
9BPV1	6.8×10^5	4.1×10^3 (0.61)
10CPF1	1.3×10^5	1.6×10^4 (1.18)
14BPV2	5.3×10^5	3.2×10^3 (0.61)
15BWV3	1.2×10^5	6.3×10^2 (0.53)
16BPM1	2.6×10^6	2.6×10^4 (0.99)
19BWM2	1.9×10^6	6.6×10^3 (0.35)
23APV1	3.1×10^6	1.4×10^4 (0.44)
24BWM1	9.6×10^5	1.7×10^3 (0.18)
Average	1.1×10^6	6.0×10^3 (0.54)
High bacteria group		
Samples name*	Number of total bacteria per cm^2	Number of FBB per cm^2 (%)
1APG3	2.4×10^7	3.1×10^4 (0.13)
7APV1	5.4×10^6	1.0×10^5 (1.9)
11CPV3	7.6×10^7	6.1×10^4 (0.08)
12BPG3	7.7×10^6	3.2×10^4 (0.42)
13BWV1	1.6×10^7	8.3×10^4 (0.53)
17BWV1	6.2×10^6	2.8×10^4 (0.45)
18BWV1	5.7×10^6	7.5×10^5 (0.13)
20BWV1	6.0×10^6	3.5×10^4 (0.58)
21BWG2	3.9×10^7	1.5×10^4 (0.04)
22BWM1	5.6×10^6	6.2×10^3 (0.11)
25CWV1	1.3×10^8	1.9×10^5 (0.15)
26AWV1	7.3×10^6	1.6×10^5 (2.15)
27BWM2	2.2×10^7	6.3×10^4 (0.29)
Average	2.6×10^7	6.2×10^4 (0.24)

Samples are labelled based on the following abbreviation (from left-to-right):
Sample number = 1 to 27 (without sample 4)
Premise grades = A (clean), B (moderately clean), C (not clean);
Types of CB = P (plastic), W (wood);
Usages of CB = G (common), M (chicken/beef), F (fruit), V (vegetables);
Physical conditions = 1 (clean), 2 (average), 3 (dirty)

5.3.5 Multiple regression analysis

Multiple regressions were performed to determine the ecological relation of FBB and different bacterial phyla (Table 5.4), classes (Table 5.5), orders (Table 5.6) and families (Table 5.7). In this analysis, only orders in high bacteria group showed the significant result (*p* value= 0.020). The equation for the regression line was

y = 3.511 – 0.970 (*Aeromonadales*) – 0.462 (Bacillales) + 0.617 (Burkholderiales) + 0.057 (Clostridiales) – 0.016 (Enterobacteriales) – 0.230 (Flavobacteriales) – 0.037 (Lactobacillales) + 0.019 (Pseudomonadales) – 0.064 (Spingobacteriales) – 0.451 (Spingomonadales) + 0.091 (Xanthomonadales)

Table 5.4 Multiple linear regression of factors (phyla) related to the percentage of FBB

	High bacteria group				Low bacteria group			
	Unstandardized coefficient		Standar-dized coefficient		Unstandardized coefficient		Standar-dized coefficient	
Variables*	B	SE	β	*p* value	*B*	SE	β	*p* value
Constant	2.336	11.373		0.844	.519	1.056		0.641
Actinobacteria	-.035	.130	-0.283	0.796	.078	.044	0.473	0.130
Bacteroidetes	-.041	.115	-0.748	0.737	.012	.014	0.443	0.442
Cynobacteria	-1.985	2.854	-0.316	0.513	-1.201	.778	-0.598	0.173
DeinococcusThermus	.039	1.438	0.010	0.979	-.340	1.044	-0.110	0.756
Firmicutes	-.026	.116	-1.057	0.833	.010	.019	0.247	0.634
Proteobacteria	-.011	.118	-0.402	0.930	-.003	.011	-0.166	0.773

* *Bacteroidetes, Firmicutes* and *Proteobacteria* account for more than 92% of all samples.

Table 5.5 Multiple linear regression of factors (classes) related to the percentage of FBB

	High bacteria group				Low bacteria group			
	Unstandardized coefficient		Standar-dized coefficient		Unstandardized coefficient		Standar-dized coefficient	
Variables*	B	SE	β	*p* value	*B*	SE	β	*p* value
Constant	3.942	12.622		0.767	1.606	1.653		0.376
Actinobacteria	-.010	.112	-0.085	0.929	.064	.112	0.387	0.595
Alphaproteobacteria	-.075	.237	-0.605	0.765	-.026	.059	-0.322	0.682
Bacilli	-.041	.129	-1.657	0.763	-.011	.029	-0.289	0.734
Clostridia	-.053	.116	-0.413	0.664	-.060	.060	-0.506	0.357
Flavobacteria	-.091	.199	-0.764	0.666	-.003	.023	-0.127	0.890
Gammaproteobacteria	-.028	.132	-1.060	0.840	-.013	.017	-0.722	0.481

* Few orders with high bacteria count were continued for analysis.

Table 5.6 Multiple linear regression of factors (orders) related to the percentage of FBB

	High bacteria group				Low bacteria group			
	Unstandardized coefficient		Standar-dized coefficient		Unstandardized coefficient		Standar-dized coefficient	
Variables*	B	SE	β	*p* value	*B*	SE	β	*p* value
Constant	3.511	.112		0.020	.988	1.493		0.628
Aeromonadales	-.970	.022	-1.492	0.015*	.175	.321	0.409	0.683
Bacillales	-.462	.012	-1.385	0.016*	-.009	.033	-0.203	0.827
Burkholderiales	.617	.012	0.861	0.013*	-.719	.931	-1.032	0.581
Clostridiales	.057	.007	0.440	0.077	-.031	.071	-0.260	0.738
Enterobacteriales	-.016	.001	-0.595	0.033*	-.003	.016	-0.173	0.899
Flavobacteriales	-.230	.005	-1.921	0.013*	.004	.022	0.146	0.886
Lactobacillales	-.037	.001	-1.542	0.021*	.018	.091	0.197	0.878
Pseudomonadales	.019	.004	0.212	0.141	-.008	.014	-0.600	0.673
Spingobacteriales	-.064	.001	-0.822	0.011*	-.058	2.681	-0.019	0.986
Spingomonadales	-.451	.022	-0.441	0.031*	.056	.151	0.324	0.774
Xanthomonadales	.091	.004	0.531	0.029*	.164	.878	0.157	0.883

F-test for high bacteria group give significant result (*p* value= 0.020)
* Several families in the orders with *p* value less than 0.05 were continued for regression analysis

Table 5.7 Multiple linear regression of factors (families) related to the percentage of FBB

	High bacteria group				Low bacteria group			
	Unstandardized coefficient		Standardized coefficient		Unstandardized coefficient		Standardize d coefficient	
Variables*	B	SE	β	*p* value	*B*	SE	β	*p* value
Constant	1.869	.654		0.214	.025	.083		0.814
Aeromonadaceae	.394	.333	0.606	0.447	.466	.101	1.094	0.136
Bacillaceae	.004	.949	0.002	0.997	.172	.247	0.656	0.613
Carnobacteriaceae	22.365	10.203	1.715	0.272	-6.096	3.408	-2.121	0.325
Comamonadaceae	-15.280	35.785	-0.205	0.743	-115.694	32.795	-0.835	0.176
Enterobacteriaceae	.007	.008	0.256	0.525	.009	.001	0.618	0.096
Enterococcaceae	.234	.303	0.282	0.581	.334	.161	2.922	0.286
Flavobaceriaceae	-.220	.085	-1.836	0.234	.003	.005	0.106	0.667
Lactobacillaceae	-1.606	.609	-2.376	0.231	-2.106	.947	-0.769	0.269
Leuconostocaceae	.004	.008	0.149	0.717	-.099	.193	-0.507	0.698
Streptococcaceae	-1.842	1.164	-0.728	0.359	3.948	.806	1.640	0.128
Xanthomonadaceae	.221	.089	1.288	0.242	-.311	.181	-0.298	0.335

[1] Analysis was limited for 11 variables only.
[2] Genera with β value more than 0.5 and less than -0.5 was continued for correlation analysis. Regression analysis for genera was not performed due to high number of variables

5.3.6 Comparing the population of FBB and 11 orders of bacteria

The comparison of 11 orders of bacteria and 12 species of FBB are shown in Figure 5.1. Foodborne bacteria were dominated by *Cronobacter sakazakii* and *Salmonella enterica*, with few samples that contained a high number of *Escherichia coli* (6BWG1, 26AWV1 and 27BWM2). Low and high bacteria group were dominated by *Enterobacteriales, Flavobacteriales* and *Pseudomonadales* with an addition of *Lactobacillales,* in high bacteria group. Moreover, low bacteria group contained a higher percentage of *Pseudomonadales* as well as *Enterobacteriales* (Figure 5.2 A), but a lower percentage of these orders in high

bacteria group. In high bacteria group, *Lactobacillales* were recorded to be higher than that of low bacteria group (Figure 5.2 B).

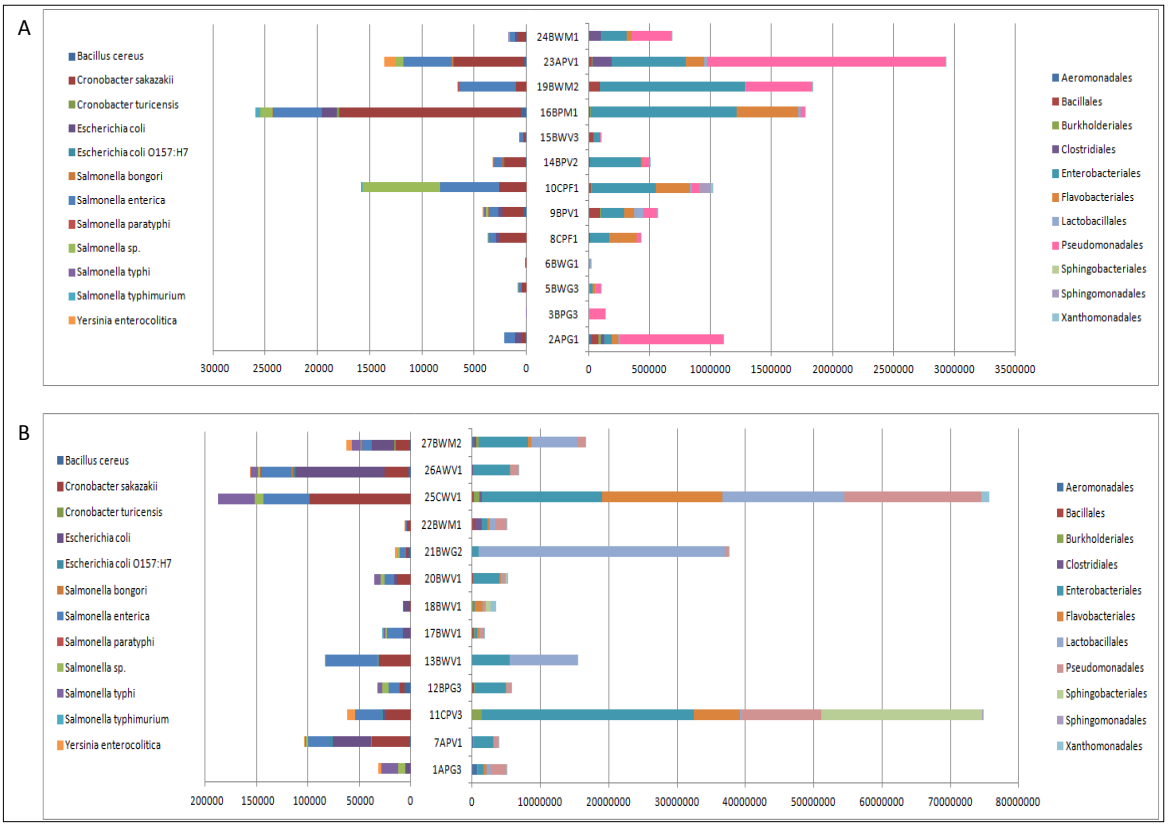


Figure 5.1 Comparison of the number of FBB and orders of bacteria in low (A) and high bacteria group (B). Note that the values of the x-axis are not the same.

5.3.7 Bacterial orders in low and high bacteria group

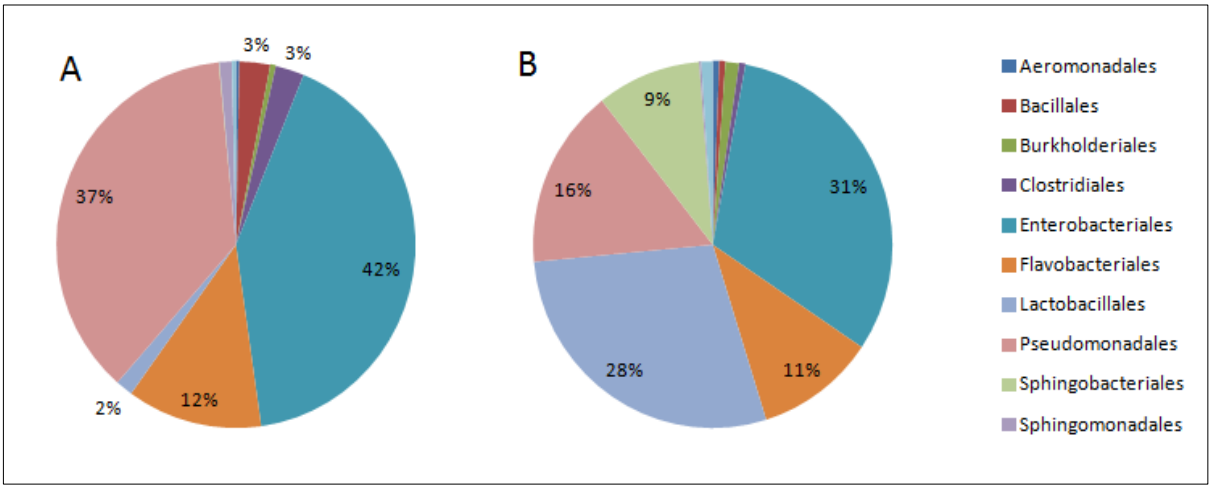


Figure 5.2 Comparison of bacterial orders in low (A) and high (B) bacteria group

Further analysis was done to determine the association of FBB and orders in high bacteria group. This is based on regression analysis, which pointed out a significant ecological correlation between high bacteria group and FBB. Correlation analysis of these orders showed that only *Enterobacteriales* had significant positive correlation with FBB (Table 5.8). None of a single bacterial order had significant negative correlation with FBB, thus, several combination of these orders were further tested. The result showed that the combination of *Clostridiales, Flavobacteriales* and *Lactobacillales,* had a significant negative association with FBB (r = -0.583, *p*-value = 0.036) (Figure 5.3).

5.3.8 Correlation analysis

Table 5.8 Correlation between FBB and orders in high bacteria group

Bacterial orders[a]	*r*	*p*-value
Aeromonadales	0.280	0.354
Bacillales	0.236	0.437
Burkholderiales	0.165	0.590
Clostridiales	-0.050	0.875
Enterobacteriales	0.692	0.009*
Flavobacteriales	-0.269	0.374
Lactobacillales	-0.231	0.448
Pseudomonadales	-0.088	0.775
Spingobacteriales	0.115	0.707
Spingomonadales	0.393	0.184
Xanthomonadales	0.280	0.354

* Correlation is significant at the 0.05 level (2-tailed).
[a] Combination of more than 1 orders was continued for analysis

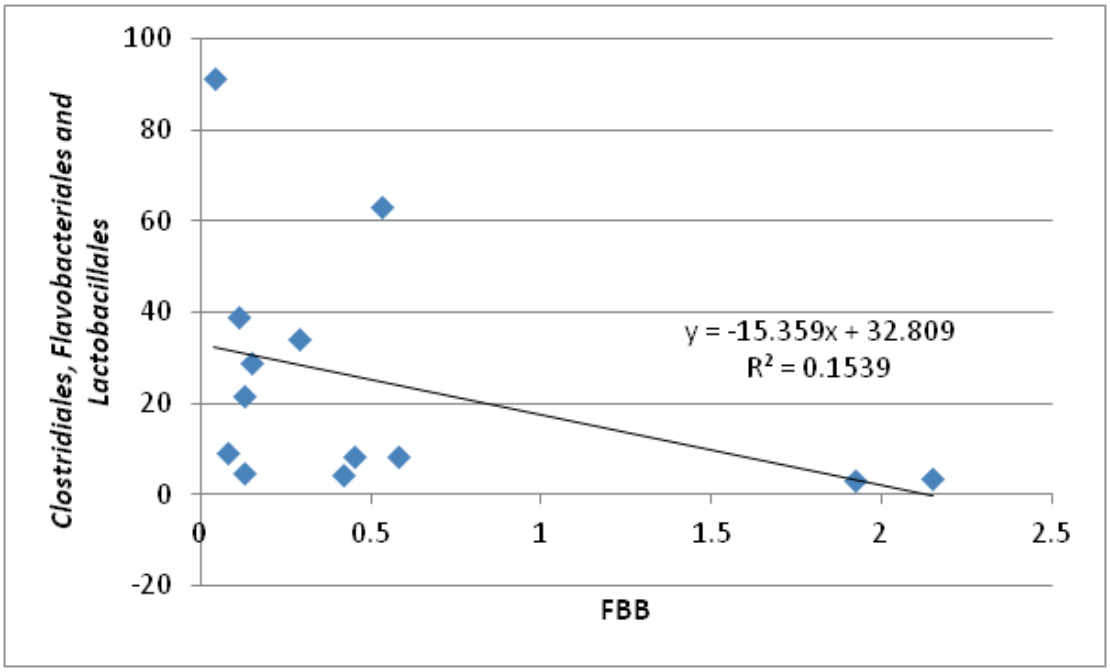


Figure 5.3 Scatter plot of the percentage of *Clostridiales, Flavobacteriales, Lactobacillales,* and FBB in high bacteria group (r = -0.583, *p* value = 0.036).

In addition, regression analysis for families showed high β value for *Carnobacteriaceae, Flavobaceriaceae, Lactobacillaceae* and *Xanthomonadaceae* in high bacteria group; and *Aeromonadaceae, Carnobacteriaceae, Enterobacteriaceae, Enterococcaceae* and *Streptococcaceae* in low bacteria group (Table 5.7). The genera in these families were used for correlation analysis which showed a significant positive correlation between FBB and 4 genera (*Citrobacter*, *Enterobacter, Erwinia* and *Klebsiella*) in high bacteria group and 3 genera (*Citrobacter, Enterobacter* and *Pantoea*) in low bacteria group (Table 5.9). All of these genera belong to the *Enterobacteriaceae* family.

Table 5.9 Correlation between FBB and genera belong to family with high β value

	High bacteria group		Low bacteria group	
	r	*p*-value	*r*	*p*-value
Acidovorax	-0.137	0.654	-0.136	0.658
Aerococcus	-0.182	0.552	0.154	0.615
Aeromonas	0.280	0.354	-0.198	0.517
Alkalibacterium	0.386	0.193	0.236	0.438
Anoxybacillus	NA	NA	-0.309	0.305
Aquabacterium	-0.197	0.519	0.077	0.802
Atopostipes	-0.409	0.116	0.026	0.932
Bacillus	0.442	0.130	-0.309	0.304
Buttiauxella	0.025	0.935	0.075	0.807
Candidatus	0.227	0.456	-0.496	0.085
Carnobacterium	-0.476	0.100	-0.463	0.111
Cedecea	0.386	0.193	0.386	0.193
Citrobacter	0.648	0.017*	0.714	0.006*
Comamonas	0.072	0.816	-0.138	0.653
Curvibacter	0.077	0.802	-0.201	0.510
Dickeya	0.197	0.519	NA	NA
Dolosigranulum	0.077	0.802	0.154	0.615
Enterobacter	0.791	0.001*	0.659	0.014*
Enterococcus	-0.258	0.394	-0.126	0.681
Erwinia	0.681	0.010*	0.007	0.803
Ewingella	0.251	0.408	-0.034	0.913
Exiguabacterium	-0.102	0.741	0.156	0.611
Flavobacterium	0.337	0.260	-0.055	0.858
Hafnia	-0.022	0.943	-0.050	0.872
Hydrogenophaga	0.003	0.992	0.231	0.447
Ideonella	0.390	0.188	NA	NA
Klebsiella	0.659	0.014*	0.527	0.064
Kluyvera	0.319	0.289	0.319	0.289
Lactobacillus	-0.368	0.216	-0.118	0.700
Lactococcus	-0.181	0.553	0.457	0.117
Leptothrix	-0.376	0.205	-0.376	0.205
Leuconostoc[LAB]	-0.148	0.629	0.176	0.556
Limnohabitants	-0.231	0.447	NA	NA
Marinilactibacillus	NA	NA	0.022	0.942
Morganella	-0.142	0.643	0.014	0.964
Obesumbacterium	0.059	0.847	NA	NA
Oceanobacillus	-0.070	0.821	0.254	0.402
Ornithinibacillus	NA	NA	-0.154	0.615
Pantoea	0.330	0.271	0.588	0.035*
Pectobacterium	-0.154	0.616	0.424	0.149
Pediococcus[LAB]	0.130	0.672	0.275	0.363
Proteus	0.427	0.145	-0.231	0.447
Providencia	0.212	0.487	0.212	0.487
Rahnella	-0.143	0.642	-0.273	0.367
Raoultella	0.336	0.262	0.547	0.053
Roseateles	-0.183	0.549	NA	NA
Serratia	0.357	0.231	0.254	0.402
Solibacillus	NA	NA	-0.077	0.802
Streptococcus	0.283	0.350	0.270	0.372
Streptomyces	0.302	0.316	-0.372	0.211
Vagococcus	-0.100	0.745	-0.416	0.157
Variovorax	-0.048	0.877	0.070	0.821
Virgibacillus	NA	NA	0.154	0.615
Weisella[LAB]	-0.088	0.775	0.170	0.578
Xenorhabdus	0.386	0.193	0.077	0.802

* Correlation is significant at the 0.05 level (2-tailed).

NA = Not applicable

5.4 Discussion

5.4.1 The effect of background bacteria on FBB growth

Pyrosequencing analysis has been widely used to study microbial community in various samples like abomasal ulcers (Hund *et al*., 2015), blood cultures (McCann *et al*., 2015), drinking water distribution system (Prest *et al*., 2014) and refrigerator (Jeon *et al*., 2013). Based on our understanding, this is the first application of pyrosequencing used to characterize microbial communities in CB samples collected from food service establishments. The analysis showed that there was no significant difference in total bacterial number based on types, usages and conditions of CB. This event occurred probably due to the constant hot temperature, especially in the kitchen area which favours the growth of all microorganisms regardless of the types of CB used, as well as the type of food being handled on the CB. However, statistical analysis showed that premise grades C contained significantly higher total bacterial number compared to premise grades A and B. The low level of hygiene in premise grade C might allow for all types of microorganisms to grow including harmless bacteria.

As mentioned earlier, 5.0×10^6 bacterial cells/cm^2 was chosen as a threshold for low and high bacteria groups. This number was in agreement with Jay (1996) who stated that the high population of surrounding bacteria can affect the growth of pathogenic bacteria. This means that high total number of bacteria can help to ensure a safe surrounding and avoid the growth of bad bacteria. In this study, we found that the percentage of FBB was higher in low bacteria group compared to that of in high bacteria group, which probably indicates that the total number of bacteria could influence bacterial number as well as their interaction. In this study, bacteria that had a significant association with FBB were different in high and low bacteria group. Even though the number of some FBB in the high bacteria group were significantly higher than that of low bacteria group based on premises, types, usages and conditions of CB, they did not exceed the infectious dose level. Infectious dose of foodborne bacteria differ from one species to another, for example, 10^5 organisms of *Salmonella* and *E. coli* (Kothary and Babu, 2001), 700 organisms of *E. coli* O157:H7 (Tuttle *et al*., 1999), 10^4 to 10^5 CFU/ml of *Bacillus cereus* (Pina-Pérez *et al*., 2012), 10 CFU/g of *Cronobacter sakazakii* (Fakruddin *et al*., 2013) and 10^5 to 10^6 cells of *Yersenia enterocolitica* (Sreedharan *et al*., 2012).

5.4.2 Ecological relationship of bacterial orders and FBB

The significant ecological relationships between bacterial orders and FBB were analyzed further. Most of these bacteria can be easily found in the environment and are usually harmless. However, certain species can cause diseases in animals, plants as well as humans. Generally, *Aeromonadales* are aquatic inhabitants, and pathogens in this order are *Aeromonas hydrophila* and *A. caviae* (Martin-Carnahan and Joseph, 2005). Bacteria in the *Bacillales* order like *Bacillus cereus* and *B. anthracis* can produce spore to survive harsh conditions (Paredes-Sabja *et al*., 2011) and cause diseases. *Burkholderiales* comprise of bacteria that can cause respiratory infections (*Bordetella* spp.) and

granulomatous disease (*Burkholderia cepacia* complex spp.) (Sim *et al*., 2010). *Sphingobacteriales* and *Sphingomonadales* are soil and marine microorganisms respectively (Domínguez-Mendoza *et al*., 2014; Zheng *et al*., 2011; Kim and Kwon, 2010), whereas *Xanthomonadales* contain many pathogenic bacteria that affect humans, animals and plants (Cutiño-Jiménez, 2010). Most of these orders of bacteria are Gram-negative and it was reported that the emergence of Gram-negative pathogens which are resistant to antimicrobial agents have increased (Nordmann *et al*., 2011).

This study identified the possible interaction of bacteria that suppressed and supported bacteria growth. Since bacteria grow in complex communities comprising other species of bacteria, we did a few statistical tests to determine which bacteria can help to suppress the growth of FBB. This is based on the fact that bacteria interact with each other in human body, especially in the intestine to ensure the health and keeping a low number of harmful microorganisms (Vogt *et al*., 2015; Shoaie *et al*., 2013; Hooper *et al*., 2002). The interaction might also occur in the environment which is the base of our study. The result showed that the combination of a few orders of bacteria like *Clostridiales, Flavobacteriales,* and *Lactobacillales* was found to have a significant negative association with FBB.

Clostridiales composed of bacteria in the *Firmicutes* phylum*,* which is also a predominant order in the healthy gut (Abell and McOrist, 2007). Orders *Clostridiales* and *Lactobacillales* are microbiota of healthy individual and are considered autochthonous/good bacteria (Thiennimitr *et al*., 2012, Rai *et al*., 2015). Many members of this order like *Clostridium* and *Bacillus* can produce spores (Paredes-Sabja *et al*., 2010). However, not all members of this order are harmful; for instance, *Caprococcus* were found to benefit the host especially when supported by other bacteria from the order *Lactobacillales* (Ferrario *et al*., 2014). A study by Baumgart *et al.,* (2007) discovered that a reduced population of *Clostridiales* can enhance the growth of *E. coli* implying the significance of this *Clostridiales* order to suppress bad bacteria*. Clostridiales* order is also essential to impair the colonization of pathogenic bacteria like *Clostridium difficile, E. coli* and *Salmonella* (Stecher and Hardt, 2011; Reeves *et al,* 2012; Kamada *et al*., 2013; Vincent *et al.* 2013) suggesting the importance of *Clostridiales* to reduce the growth of bad bacteria.

The order *Clostridiales* was discovered to be producing *comQXPA* a QS system important in encoding four proteins, which are isoprenyl transferase, pre-peptide signal, histidine kinase, and a response regulator. Upon reaching a specific concentration of these molecules a large number of cellular responses will be activated for competence development, surfactin production, biofilms formation and extracellular DNA release (Dogsa *et al.,* 2009), thus, allow for these bacteria to compete with pathogenic bacteria and impair their growth. Few bacterial species in *Clostridiales* order could also produce *luxS* gene to synthesis autoinducer-2 (AI-2), an important signalling molecule for the production of biofilms, which ensure bacterial survivability (Pantaléon *et al*., 2014).

Flavobacteriales is located in the phylum of *Bacteroidetes*. In humans, *Flavobacteriales* reside on dry sites of the skin along with β-*Proteobacteria* (Schommer and Gallo, 2013). They are also ubiquitous in soil and are usually associated with plant rhizosphere and phyllosphere (Kolton *et al*., 2013). *Flavobacteriales* are one of the beneficial microbial communities to ensure plant health and soil fertility, and can be easily isolated from soil and water (Schlaeppi *et al*., 2013; Chen *et al*., 2014). Flavobacteria are known to possessed antimicrobial components and demonstrate antagonistic properties towards other bacteria (Grossart *et al*., 2004). Flavocin, an agent with fungisitic and bacteriostatic activities produced by *Flavobacterium* sp. L-30 is widely used to treat various farm crops (IuD *et al*., 1996). Tyc *et al*., 2014 discovered the antimicrobial activity of Flavobacteria against *S. aureus* and *E. coli* and the interaction was enhanced with the combination of alpha-Proteobacteria. In another study, Goecke *et al*. (2012) revealed an interesting finding on several bacteria including *Flavobacteria* that demonstrates antibiotic activity against microorganisms like *Bacillus subtilis, Candida glabrata*, *Escherichia coli* and *Staphylococcus lentus.* They also stated that there were complex chemical interactions that influenced the relationship of the bacteria.

Flavobacteriales, specifically *Chryseobacterium* was discovered to be producing *aidC* gene that has *N*-Acylhomoserine lactones (AHLs) degrading activity. AHLs are QS signal molecules used many Gram-negative bacteria. A study by Wang *et al*. (2012) concludes that *Chryseobacterium* from *Flavobacteriales* order, produce Aidc which functions as AHL lactonase, catalyzing AHL ring opening by hydrolyzing lactones. Few studies have identified the significance of quorum sensing inhibition or quorum quenching by identifying as well as developing chemical compounds and enzymes that targeting signaling molecules, signal biogenesis, or signal detection. The therapies are effective against some pathogens but still require further studies (LaSarre and Federle, 2013).

Lactobacillales comprise of lactic acid bacteria which are usually associated with fermentation and human nutrition (Salvetti *et al*., 2013). Generally, *Lactobacillales* or LAB are natural inhabitants of the human gastrointestinal tract (de Almeida Júnior *et al*., 2015) and some species are qualified as probiotics which have health-promoting activity (Ashraf *et al*., 2014). Cell-to-cell communication or quorum sensing (QS) between LAB occurs when a concentration of a specific molecule is reached, act as signals for induction of specific gene, thus allow for the production of bacteriocin-like nisin and lacticin (Kuipers *et al*., 1998). As they multiply, they will produce metabolites like bacteriocin (Macwana and Muriana, 2012), lactic acid and acetic acid (Vermeulen *et al*., 2007) which plays an important role in controlling food-spoilage and food-borne pathogens (Hwanhlem *et al*., 2014; Ghanbari *et al*., 2013). In addition, plantaricin and weisellicin, two types of bacteriocins produced by *Lactobacillus plantarum* and *Weisella hellenica* respectively, were found to be active against foodborne bacteria like *S. aureus, L. monocytogenes, B. cereus, E. coli, Clostridium perfringens, Salmonella typimurium,* few Gram-positive bacteria as well as some yeasts and molds (Chen *et al*., 2014; da Silva Sabo *et al*., 2014; Leong *et al*., 2013).

5.4.3 Correlation of bacterial genera and FBB

The correlation analysis showed that bacteria in the genera of *Citrobacter, Enterobacter, Erwinia, Klebsiella,* and *Pantoea* had a significant relationship with FBB. They, along with *Cronobacter, Escherichia and Salmonella* are in the *Enterobacteriaceae* family as well as the *Enterobacteriales* order (Baylis *et al*., 2011). This explains the positive relationship of *Enterobacteriales* and FBB. *Escherichia coli, Shigella, Salmonella* and *Yersinia* are usually associated with the intestines and faeces of mammals and birds (Feng *et al*., 2013). Bacteria in this group are also known as the most common human pathogens, causing infections in the community as well as in hospitals (Nordmann, 2014). Some species in the *Enterobacteriaceae* family like *Escherichia coli, Salmonella, Shigella, Yersinia entercolitica,* and *Enterobacter* can cause diseases and infections in the gastrointestinal tract (Singh *et al*., 2015). Bacteria in these families are Gram-negative microorganisms and are usually associated with community-acquired and healthcare-associated infections (Gharout-Sait, 2015).

Citrobacter and *Enterobacter* are microflora within the human gut but could cause infection in immunocompromised persons (Algubaisi *et al*., 2015; Chen *et al*., 2014; Lai *et al*., 2010). *Enterobacter* is microflora of the gastrointestinal tract, but under some circumstances, they can cause pneumonia, urinary tract infections, wound infections and bacteraemia (Chen *et al*., 2014). *Erwinia* is known as a plant pathogen which infects pear and apple trees (Hannou *et al*, 2013; Gehring and Geider, 2012). *Klebsiella* is widely distributed in the environment and intestinal flora of humans and mammals (Santos *et al*., 2011). In addition, *Klebsiella pneumonia* is an opportunistic pathogen causing pneumonia (Marques *et al*., 2010) and could also cause a liver abscess (Wu *et al*., 2015). *Klebsiella rhinoscleromatis* and *K. ozaenae* are two other species that could cause diseases of the upper airways (Botelho-Nevers *et al*., 2007). *Pantoea* usually inhabits plants, soil and water, and several species of *Pantoea* have been reported to cause diseases in humans and plants such as bacteraemia (Cheng *et al*., 2013) neonatal sepsis; a blood infection in infants (Mehar *et al*., 2013) and bacterial disease of sweet corn and maize (Ammar *et al*., 2014).

Bacteria within *Enterobacteriaceae* family could interact with each other via QS to ensure the survivability of their species; which might explain their positive correlation with FBB. Bacteria in these families are Gram-negative microorganisms and are usually associated with community-acquired and healthcare-associated infections (Gharout-Sait, 2015). A positive correlation between several genera and FBB might be due to the interspecies interaction that supports their growth. The interaction could occur via quorum sensing.

In Gram-negative bacteria, N acyl-L-homoserine lactones (AHLs) represent the most intensively studied family of signal molecules (Winzer and William, 2001). With the increase of this molecule, microorganisms can cause diseases, form biofilms, and compete with other types of bacteria (Gobbetti *et al*., 2007; Winzer and William, 2001). Different species of bacteria communicate through AI-2 (Rui *et al*., 2012). Using AI-2, bacteria are capable of detecting the presence of other species of bacteria in their surrounding and regulate gene expression according

to the species composition (Ascenso *et al*, 2011). Thompson *et al*. (2015) discovered that AI-2 produced by engineered *E. coli* help to increase the number of *Firmicutes* in the gut. Walters and Sperandio (2006) also stated that intestinal bacteria like *Salmonella* and *E. coli* use this interspecies signalling molecule as a way to enhance virulence gene expression and host colonization. In another study by Dourou *et al*. (2011), the growth of *Salmonella* was believed to be supported by the production of a signalling compound of *Yersinia enterocolitica* and *Serratia proteamaculans*.

The findings strengthen the fact that different bacteria can support the growth of each other. The same signalling molecules allow for the interspecies communication, thus in this study, it was observed that the increase in a number of pathogenic bacteria increases the number of foodborne bacteria as well.

Although the major finding from this study discovered that, high background biota can impair the growth of pathogenic bacteria; it should not be used to measure the likelihood of foodborne disease. This is because there are a lot of factors that should be taken into account for a foodborne disease to occur, for example, infectious dose level of FBB, high-risk population, human health condition as well as the microflora in the gastrointestinal system (Foodborne Illness Investigations and Control Reference Manual, 1996).

5.5 Conclusion

This study reveals a high diversity of microorganisms including FBB present in CB samples. Premise grade C contains a significantly higher number of total bacteria compared to premise grades A and B, and in certain conditions and surfaces, high bacteria group contain a significantly higher number of FBB compared to low bacteria group, however, not exceeding the infectious dose to cause illness. In overall, high bacteria group contain low prevalence of FBB compared to low bacteria group. The significant negative association was discovered between FBB and the combination of *Clostridiales, Flavobacteriales,* and *Lactobacillales* in high bacteria group. Additionally, 4 genera (*Citrobacter, Enterobacter, Erwinia* and *Klebsiella*) in high bacteria group and 3 genera (*Citrobacter, Enterobacter* and *Pantoea*) in low bacteria group had significant positive correlation with FBB suggesting the probability of their interaction in supporting the number of harmful microorganisms.

In short, the population of overall bacteria does have an advantage of reducing the number of FBB. This study also suggested that microbial interaction on the CB samples were very diverse and some genera might have the ability to reduce and support the growth of FBB. Further investigation specified on the bacteria can be done to study their relationship with FBB, possible interaction and factors that affect the number of pathogens.

CHAPTER 6: CONCLUDING REMARKS, AND RECOMMENDATIONS FOR FUTURE RESEARCH

6.1 Conclusion

Pyrosequencing is one of the molecular techniques, widely used to study microbial communities from various samples. However, this is the first study that used the pyrosequencing technique on cutting board samples collected from food service establishments.

This study reveals a high diversity and high number of microorganisms with an average count of bacteria of 1.4×10^7 bacterial cells/cm^2. The samples were dominated mainly by the phyla of *Proteobacteria* and *Firmicutes.* In addition, genera *Pseudomonas, Enterobacter, Acinetobacter* and *Weisella* were found in most samples.

This study also shows that level of cleanliness did not associate with species richness of bacteria as well as the species of FBB reside on the CB. This indicated that CB in less clean premises does not necessarily contain more diverse microorganisms compared to cleaner premises. Moreover, few numbers of foodborne bacteria were also found in this study. Surprisingly, the percentage of foodborne bacteria in some grade A premises were higher compared to lower premise grades and they contain dangerous bacteria like closely related species to *E. coli* O157:H7 and *Salmonella.* This finding points out that clean environment did not guarantee food that is free of dangerous bacteria. Although these pathogenic bacteria were isolated from the surface of the cutting board and not from the food samples, food handlers can still cause foodborne disease if they ignore correct food handling measures and cross contaminate food items.

This study shows that plastic and wood CB; whether they are used to cut chicken, beef, vegetables or fruit; and whether they look clean or dirty, they have equal chances of introducing FBB to food items. Subsequent analysis also reveals another set of information. In samples with a high number of background bacteria, the percentage of FBB was found to be lower compared to samples with low background bacteria. Multiple regression analysis showed that, in high background biota, bacterial orders significantly correlated with the FBB and the combination of *Clostridiales, Flavobacteriales,* and *Lactobacillales* had the potential to reduce FBB growth. This is because, as the growth of these three bacterial orders increases, the growth of FBB decreases. This shows that more than a group of bacteria with different characteristics work synergistically to ensure their suppressive effect towards FBB.

However, as oppose to this, bacteria like *Citrobacter, Enterobacter, Erwinia, Klebsiella* and *Pantoea* from the *Enterobacteriaceae* family had significant positive correlation with FBB. Some of the members in these bacteria genera are associated with diseases and their interaction in supporting the growth of each other might be dangerous and should be a concern. Although this is the case, high background biota can ensure that the population of harmful bacteria are kept low and fail to reach the infectious dose level, thus fail to cause illness.

The fact that FBB can be found in high grades of food premises and all types, usages and conditions of CB indicate that consumers facing the same chances of getting illness wherever possible. Although this study discovered the ability of background bacteria to suppress the growth of foodborne pathogens, it does not necessarily mean that dirty condition can give a great advantage to premises. In conditions that favour FBB growths, the risk would be greater and possible for the foodborne outbreak to occur. The fact that clean premises also harbour foodborne bacteria indicates that all premises must practice correct food manipulations. Hence, food handlers hold a huge responsibility to practice correct food handling measures like washing hands and food contact surfaces when necessary, avoiding cross-contamination, ensuring right temperature is reached during cooking, storing or serving. To top it off, due to the importance of background biota, it is necessary that kitchen items be washed using normal sanitizer without the addition of any chemical and antimicrobial agent. This is to ensure that pathogenic bacteria do not become resistance which will lead to more dangerous diseases.

The authority plays an important role to ensure food service establishments abide the law regarding food safety. They can organize appropriate training periodically to old and new employees and constantly checking the cleanliness level of the premises. Hygienic practices of the food handlers can be one of the criteria for grading purposes. This will ensure that all personnel guarantee self-hygiene in the premises apart from the cleanliness of the restaurants.

In general, the result of this study can be used to represent Malaysia as a whole. This is because the food premises around Seri Kembangan, where the samples were taken running almost the same kind of business model, serving the same type of food and same operation hours. Malaysia is also having a constant warm temperature all year round which might contribute to the same bacterial community. However further study is required to confirm this statement.

6.2 Recommendations for future research

Many bacteria are able to produce metabolites to suppress the growth of pathogenic bacteria. The usage of these metabolites can be further studied and purified metabolites can be used in food materials or kitchen items, hence, increase the shelf life of food and avoiding the growth of FBB. These metabolites are much safer to humans compared to antibiotics and probably can be used to replace antibiotics in the future.

This current study was focusing on one location only, which is Seri Kembangan, Malaysia. Future research can be done by taking samples from other locations and then make a comparison of the microbial community from these places. Samples can be taken at local or international scale, which will give an interesting data of the community composition. Data from food service establishment can be compared with the home setting as well. The differences between different environments could favour the growth of different types of bacteria. The data can be used to determine the factors strongly associate with

food contamination or diseases and can be used in the field of food safety. QIIME software is a reliable tool to study the microbial diversity of many samples. In the future, it will be a good approach to use biplot function in QIIME to see the contribution of each taxonomic group to the coordination of samples in PCoA.

Food handlers have been identified as the most common reason for foodborne diseases. In certain cases, they become carriers of dangerous bacteria and without showing symptoms of illness, they can transfer these bacteria to the food and make the consumers sick. This could happen if the food handlers come from a different country with the less hygienic environment. Future research can be done by studying microbial community in the gut of immigrants (by taking stool sample) and the data can be used to increase awareness especially in the field of food service.

Future research can focus on kinetics study of various species of bacteria to determine the bacteria growth rate and difference conditions that might favour or interfere with the growth rate of FBB. For examples, the growth rate of bacteria during fermentation or in growth condition where the substrate, temperature or pH is manipulated. This data can help us to control the condition during food storage or food preparation to increase the quality of food and to provide safer food to consumers.

Future research can be done by focusing on the identification and quantification of FBB only. Bacterial quantification could be done by conventional colony count on agarose media and some other biochemical methods such as commercial available ATP test kit. The usage of more accurate materials and technique will provide extra confidence in the result, in addition to molecular technique. Furthermore, future study can also take into consideration of environmental factors that might contribute to the presence of different bacteria on the CB such as temperature, storage, detergents use and others.

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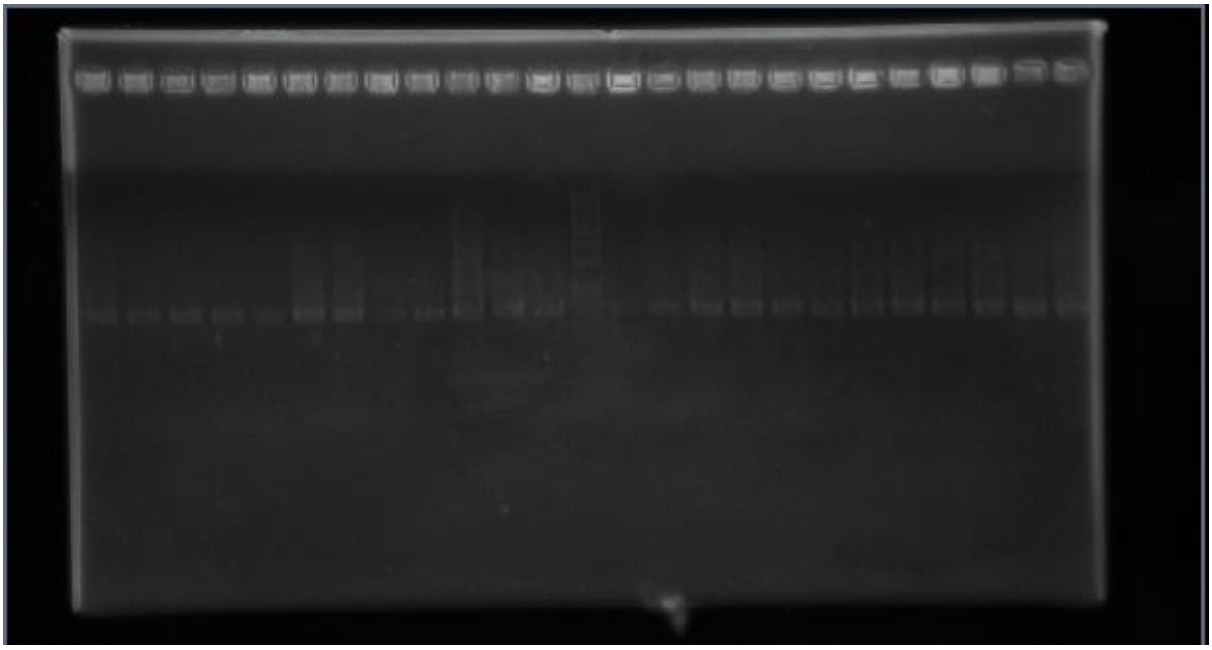
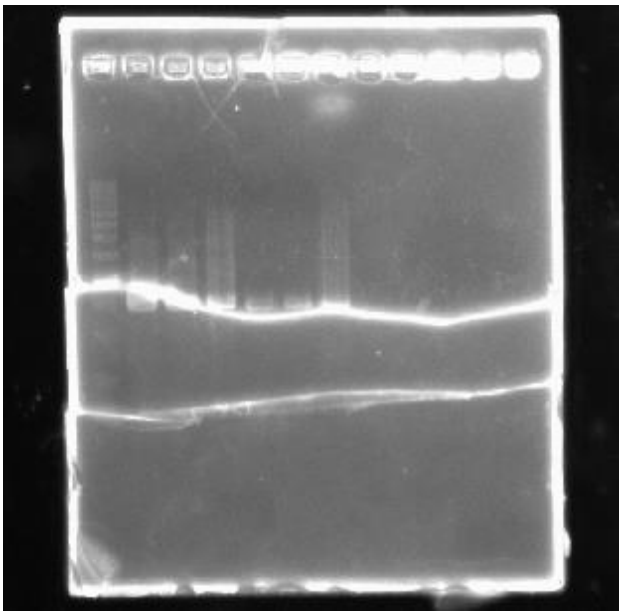
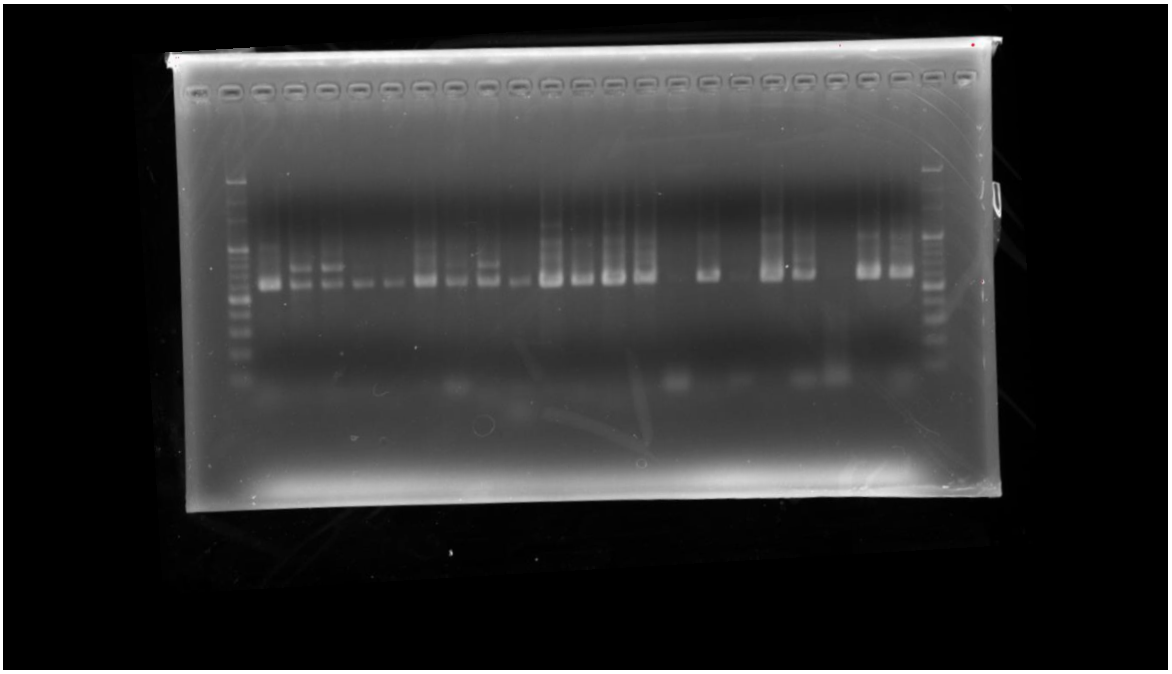
APPENDICES

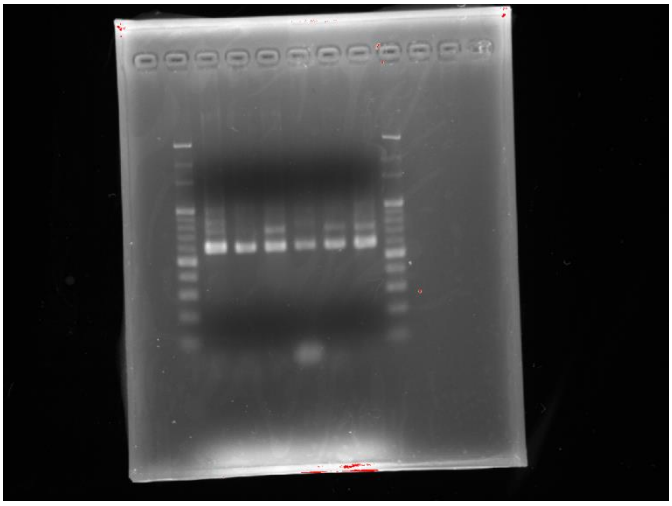
Confirmation of DNA samples extracted using kit





DNA samples after purification and PCR (used for pyrosequencing)





Analysis of pyrosequencing data

Analysis of pyrosequencing data was done using software called QIIME (Quantitative Insights into Microbial Ecology).

Firstly, Mapping File was created. The file contains information about the samples for the analysis like the name for each sample, the sequence used and the sequence of the primer for amplification. For the purpose of validation and to ensure mapping file is formatted correctly, this command was typed

```
validate_mapping_file.py –m Fasting_Map.txt -o mapping_output
```

A massage will appear indicating a problem in the mapping file. If an error was reported, correction should be made before proceeding to the next step.
The second step is to assign samples to multiplex reads based on their nucleotide barcode. This step also removed low quality or ambiguous reads. Below command was typed

```
split_libraries.py -m Fasting_Map.txt -f IQKZ9MI01.fna -q IQKZ9MI01.qual -o
split_library_output
```

New folder was created in the new directory with the name of **split_library_output/:** This folder contains three files which consist of **split_library_log.txt** which contains the summary of splitting, including the number of reads detected for each sample and a brief summary of any reads that were removed due to quality considerations. A file with the name of **histograms.txt** contain the number of reads at regular size intervals before and after splitting the library, whereas **seqs.fna** is a fasta formatted file where each sequence is renamed according to the sample it came from. The header line also contains the name of the read in the input fasta file and information on any barcode errors that were corrected.

Third step, involving a chimera check which was done using Black Box Chimera Check (B2C2). This software removed any chimera reads. Chimeras are hybrid products between multiple parent sequences that can be falsely interpreted as novel organisms, thus inflating apparent diversity (Haas et al., 2011).

Forth step was assigned for picking Operational Taxonomic Units (OTUs). Sequences with greater than 97% similarity were assigned to the same species. Following command was typed

```
pick_de_novo_otus.py -i split_library_output/seqs.fna -o otus
```

The results was created in a new folder named **otus/**. The result was generated through several steps as follow:

- Picking OTUs based on sequence similarity within the reads.
- Picking a representative sequence set, one sequence from each OTU.
- Aligning the representative sequence set.
- Assigning taxonomy to the representative sequence set.
- Filtering the alignment prior to tree building - removing positions which are all gaps, or not useful for phylogenetic inference.
- Building a phylogenetic tree.
- Building an OTU table.

Next step was entering the following command

```
biom convert –i otu_table.biom –o otu_table.txt -b
```

Since the file received was large, it was devided into four new files

```
User¥dobi¥Desktop>cd-hit-div.pl seqdevide 4.txt
```

The new files names are devided-0, devided-1, devided-2 and devided-3. To download the DNA sequence, the following command was typed into the software

```
User¥dobi¥Desktop>cd desktop
```

Clicked “enter” and typed

```
perl mori.txt seqmatch_download 0
```

The fifth step involved a similarity search by Ribosomal Database Project (RDP) of the OTUs sequence. In the database, **SEQMATCH** analysis was chosen. Then file in **.txt** format was uploaded and following options were marked

Strain:	○ Type	○ Non Type	◉ Both
Source:	○ Uncultured	◉ Isolates	○ Both
Size:	○ ≥1200	○ <1200	◉ Both
Quality:	◉ Good	○ Suspect	○ Both
Taxonomy:	◉ Nomenclatural	○ NCBI	
KNN matches:	20 ▾		

Using QIIME software, alpha diversity was computed and rarefaction curves were generated. The following command was typed into the software

```
alpha_diversity.py –h
```

Next, the following command was typed

```
alpha_rarefaction.py -i otus/otu_table.biom -m Fasting_Map.txt -
o arare -p alpha_params.txt -t otus/rep_set.tre
```

The steps involved in alpha rarefaction plots were

- Generate rarefied OTUs table.
- Compute measures of alpha diversity for each rarefied OTU table.
- Collate alpha diversity results.
- Generate alpha rarefaction plots
-

The rarefaction plots were viewed by opening the file with the name `arare/alpha_rarefaction_plots/rarefaction_plots.html`.

Final step was to compute beta diversity and generate beta diversity plots. The following command was typed into the software

```
beta_diversity_through_plots.py -i otus/otu_table.biom -m
Fasting_Map.txt -o bdiv_even146 -t otus/rep_set.tre -e 1892
```

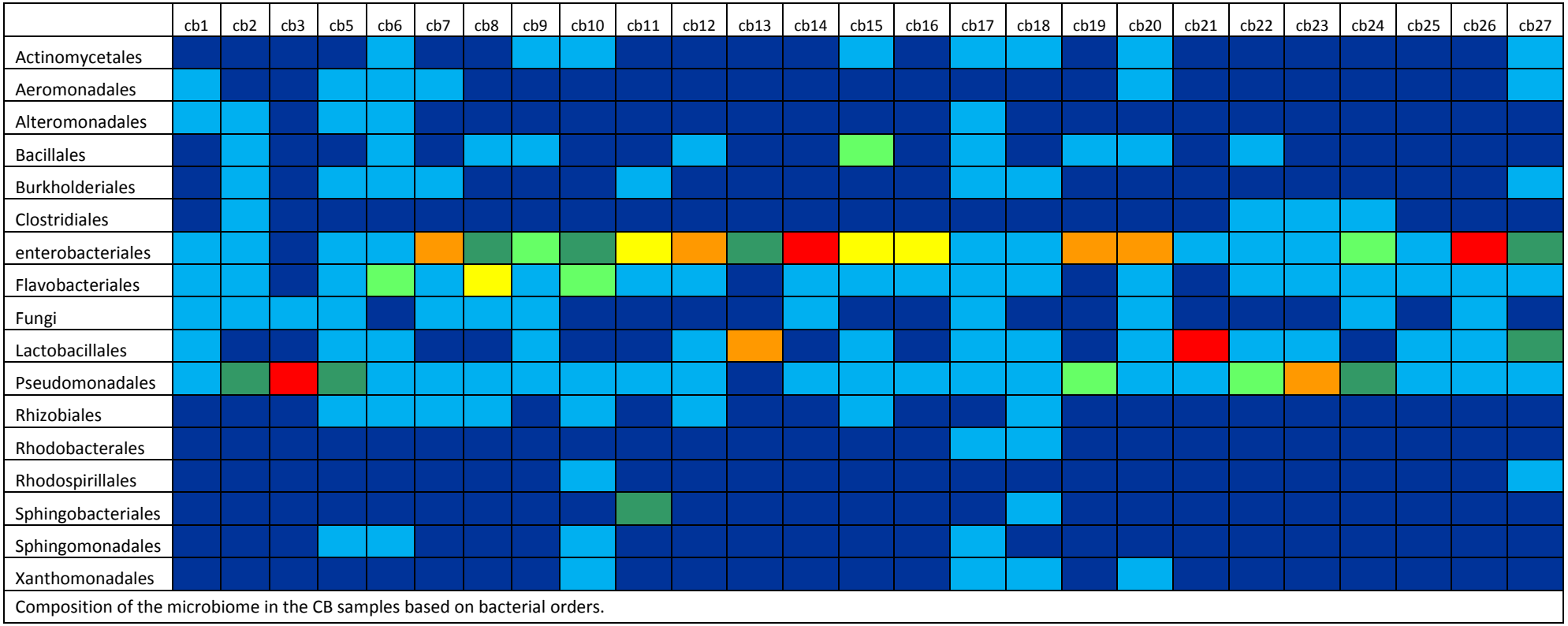
The scripts performed the following steps:

- Remove sample heterogeneity: Standardize the data obtained from samples with different sequencing efforts, and compare the OTU richness using standardize platform.
- Compute beta diversity: Assess the differences between microbial communities.
- Generates principal coordinates: Principal coordinate analysis (PCoA) helps to extract and visualize informative components of variation from complex, multidimensional data. It provides an intuitive visualization of the data structure.
- Generate emperor PcoA plots: Inspection of PCoA plots in three dimensional files.

After the process of identification finished, the file was downloaded for further analysis. The results from RDP were preferable as it cover up to species level. All of the data obtained were copied and pasted to Microsoft Excel format. Microorganisms with the same OTU were sorted and the one with the highest percentage was chosen. Then, all of the microorganisms were compiled and their total quantity was calculated.

The final step was to prepare heat map or graph based on the result using Microsoft Excel.

Heat map of bacterial orders



Composition of the microbiome in the CB samples based on bacterial orders.